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Eukaryotic Systems

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Technical Notes

UV-A, UV-B, and UV-C: This terminology, which divides the ultraviolet (UV) spectrum into three wave bands, was first proposed in 1932 by the American spectroscopist William Coblenz and his colleagues to begin to address the problem of standardizing the measurement of UV radiation used in medicine (*1,2*). Each spectral band was defined “provisionally” and “approximately” by the absorption characteristics of specific glass filters as follows: UV-A, 400–315 nm; UV-B, 315–280 nm; UV-C, <280 nm (*1*). Although based on physical specifications, these definitions were influenced by knowledge of other UV phenomenology, including biological effects and physical properties. For example, wavelengths in the UV-B band were known to have potent erythemic effects, and wavelengths below 290 nm were known to be absent from sunlight (*2*) (because they are absorbed by stratospheric ozone). Moreover, the germicidal effects of UV-C wavelengths (principally around 266 nm) from artificial sources were well-recognized (*3*). Today, the spectral bands implied by these terms may be found to vary from Coblenz’s original definitions, depending on the discipline. Environmental photobiologists, for example, generally use the following definitions: UV-A, 400–320, UV-B, 320–290, and UV-C, 290–200 (*4*).

Relative centrifugal forces: The *g*-forces listed in this book are calculated for the maximum radius unless stated otherwise. For microcentrifuges similar to Eppendorf’s 5410 and 5415 C models, maximum rotational speed (14,000 rpm) corresponds to ~12,000*g* and ~16,000*g*, respectively.

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Isolation of DNA Structure-Dependent Checkpoint Mutants in *S. pombe*

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1. Introduction

Eukaryotic cells have the ability to influence progression through the cell cycle in response to internal and external inputs of “information”. They do so by using feedback control mechanisms able to arrest mitosis in response to different cellular events. Such active mechanisms capable of influencing the timing of cell-cycle events have been called “checkpoints” (1,2). Cells arrest progression through the cell cycle if they fail to complete DNA replication or if their DNA is damaged. The S-phase/mitosis (S-M) checkpoint plays a key role in the maintenance of the interdependency between S-phase and mitosis. Wild-type *Schizosaccharomyces pombe* (fission yeast) cells arrest cell-cycle progression in response to a DNA replication block, such as that induced by hydroxyurea (HU), but continue to grow in size, since they are still metabolically active. These cells are observed to have an elongated phenotype. Mutants have been isolated in *S. pombe* that have lost the S-M checkpoint and do not prevent mitosis if DNA replication during the previous S-phase is incomplete (3–7). S-M checkpoint mutants do not delay cell-cycle events after exposure to HU, and will enter mitosis with unreplicated DNA. As a consequence, the elongated phenotype seen for wild-type cells is absent in checkpoint mutants. Instead, these mutants show a characteristic “cut” phenotype, where a cell has entered an abortive mitotic event followed by the formation of a *septum* through the nucleus. In these small dead cells, the nucleus is frequently cut in two by the septum and/or spread unevenly between both daughter cells. S-M checkpoint mutants show very low viability in the presence of HU or any other circumstance that may delay S-phase progression (e.g., in combination with a thermosensitive DNA replication mutant).

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Several of these S-M checkpoint mutants are also unable to arrest the cell cycle in response to DNA damage (6,7). The DNA damage checkpoint arrests the cell cycle in a dose-dependent manner after exposure to DNA-damaging agents. It is believed that these delays provide additional time for cells to repair the damaged genetic material before key transitions are attempted (between G1 and S-phase, G2 and mitosis, or during DNA replication). Several different mutants have been isolated in *S. pombe* that are defective in the DNA damage checkpoint. In contrast to wild-type cells, which arrest the cell cycle immediately after DNA damage (and display the previously described elongated phenotype), most of these checkpoint mutants do not show any delay in cell-cycle events after exposure to DNA-damaging agents. One or two of these mutants are only partially defective (e.g., *rad24*). As seen for the S-M checkpoint mutants, the DNA damage checkpoint mutants can also show a cut phenotype where cells enter unrestrained mitosis with damaged DNA. All these mutants are highly sensitive to DNA-damaging agents. We refer to these two checkpoints (S-M and DNA damage) as DNA structure-dependent checkpoints.

The existence of many genes whose function is required for both checkpoint controls suggests a significant overlap between these two pathways. The structural identity between the checkpoint proteins from fission and budding yeast suggests that these pathways have analogs in mammalian cells. This is supported by the growing number of human genes found to be homologous to yeast checkpoint genes (8).

We describe below methods for:

1. Generating mutants of *S. pombe*.
2. Screening those mutants for putative DNA structure-dependent checkpoint defects.
3. Distinguishing between S-M and DNA damage checkpoint deficiencies.
4. Further characterizing checkpoint mutants.

2. Materials

2.1. Media

1. Yeast extract medium (YE): 5 g/L Difco (Detroit, MI) yeast extract, 30 g/L glucose, supplemented as required with 100 mg/L leucine, adenine, lysine, uracil, and histidine.
2. Yeast extract agar medium (YEA): YE plus 20 g/L Difco agar.
3. YEP: YE plus 20 g/L Difco Bacto-peptone.
4. Phloxin B agar (YEA + P): YEA plus 0.02 mg/mL Phloxin B (Sigma, Dorset, UK). Phloxin B is stored as a stock solution at 20 mg/mL. It should be added after the medium is autoclaved and cooled.
5. YEA + P with HU: YEA + P containing 10 mM HU. HU is kept as a 1 M stock solution stored at -20°C. It is filter-sterilized and added to autoclaved, cooled medium.

2.2. Additional Reagents and Equipment

1. Ethyl methanesulfonate (EMS is listed in Sigma's catalog as methanesulfonic acid ethyl ester).
2. 254-nm UV source (e.g., germicidal lamp).
3. 4,6-Diamino-2-phenylindole (DAPI) (Sigma).
4. Calcofluor (Sigma). (Also known as "Flourescent Brightener 28.")
5. Replica plating block.
6. Whatman filters, no. 1, 150 mm. (It is not necessary to sterilize filters from a new box.)
7. Hemocytometer.
8. Light microscope equipped with a 20× long-distance working objective.
9. Fluorescence microscope.

3. Methods

3.1. EMS Mutagenesis

Optimization of the mutagenesis procedure is an empirical process. Preliminary mutagenesis studies should be performed in order to find the experimental conditions that give the highest number of potentially interesting mutants with a reasonable level of survival (not <10%). For example, a good mutagenesis procedure using wild-type cells should give approximately one DNA damage checkpoint mutant/1000 surviving cells, with a survival rate of about 10–20%. EMS has been previously used with much success for checkpoint mutant screens in fission yeast, although alternative mutagenesis procedures (e.g., using UV irradiation, *see Note 1*) should be considered, because gene targets may differ. This may be an important consideration if the desired mutants are rare or difficult to isolate.

1. Prepare a fresh 50-mL culture of log-phase cells ($OD_{600} = 0.2\text{--}0.4$) growing in YEP.
2. Collect the cells by centrifugation (~2000g) for 2 min and resuspend in 1 mL of YEP medium containing EMS (2.5–3% v/v). Ensure the EMS is completely dissolved.
3. Incubate with shaking at room temperature for 2 h.
4. Wash the cells several times with fresh medium and plate enough cells on YEA plates to give approx 500 colonies/plate. This should be around 5000 cells/plate, assuming a survival rate of approx 10%.
5. Incubate the plates at 27°C. (Different permissive conditions may be required for the isolation of thermosensitive mutants.)

3.2. Identification of S-M Checkpoint Mutants (see Note 2)

The HU sensitivity screen has been one of the most efficient and successful experimental approaches for identifying new DNA structure checkpoint mutants, since it provides easily definable phenotypes. HU is a

powerful inhibitor of the ribonucleotide reductase enzyme that catalyzes the rate-limiting step in the production of deoxyribonucleotides needed for DNA replication.

1. Replica plate the mutagenized colonies from **Subheading 3.1.** onto two plates, one YEA and one YEA + P with HU as follows: Press the master plate against the replica-plating block covered with a Whatman filter. Gently remove the plate in such way that a replica of colonies from the master plate remains on the filter. Transfer the cells to replica plates by repeating the procedure. Remove excess cells from the replica plates by pressing each plate against a clean filter. Incubate at 27°C for 24–48 h.
2. Dead colonies on HU-containing YEA + P plates will appear red in color. Phloxin B stains dead cells red, but is actively excluded from live cells. Most of these dead colonies when observed under the microscope will contain many highly elongated dead cells (stained red). The HU-sensitive S-M checkpoint mutants will have a different morphology characterized by small dead cells and no elongation.
3. From the YEA master plate, pick cells that correspond to phenotypically interesting dead colonies and patch onto a new YEA plate.
4. Confirm the phenotype of these patches by replica plating again onto YEA and YEA + P with HU. Incubate the plates at 27°C for 48 h. Discard those mutants that do not show a reproducible phenotype (*see Notes 3 and 4*).

3.3. Identification of DNA Damage Checkpoint Mutants (see Note 5)

The isolation of DNA damage checkpoint mutants is more difficult than the identification of S-M checkpoint mutants, because the phenotypes observed during the screen are not as accurate as with cells treated with HU (particularly if UV radiation is used as the selective agent). Since most S-M checkpoint mutants are also deficient in the DNA damage checkpoint, the following experimental procedure should also be used to check any new S-M checkpoint mutant previously isolated. This screen should be performed simultaneously with the HU screen by including an extra replica plating.

1. Replica-plate the mutagenized colonies onto two plates, one YEA and one YEA + P, as described in step 1 of **Subheading 3.2.** Make sure the excess of cells is removed from both plates.
2. UV-irradiate the YEA-P plates with 200 J/m².
3. Incubate at 27°C for 48 h.
4. Dead colonies on the UV-irradiated plates will appear as red “spots”. Most of these dead colonies when observed under the microscope will contain lots of dead cells (stained red), most of which will show some degree of elongation (*see step 2, Subheading 3.2.*). In the DNA damage checkpoint mutants, this elongated phenotype will be absent or greatly reduced.

5. Pick from the YEA master plate those cells that correspond to phenotypically interesting dead colonies, and patch onto a fresh YEA plate.
6. Confirm the phenotype of these patches by replica plating again onto YEA and YEA + P media. Irradiate the YEA + P plates, and incubate at 27°C for 48 h. Discard any mutants that do not show a reproducible phenotype (*see Notes 3 and 4*).

3.4. Survival Analysis

In order to have a clear picture of the nature of the mutants isolated in the screen, it is useful to determine their survival response to DNA-damaging agents and HU, and to do a microscopic analysis of cell morphology. This information will help to classify the mutants into groups, and identifies interesting and desired phenotypes.

3.4.1. HU Survival Curves (*see Note 6*)

1. Determine the cell number of a fresh exponentially growing culture using a hemocytometer.
2. Dilute to a cell density of ~5000 cells/mL in YEP.
3. Add HU to the diluted cell culture to a final concentration of 10 mM.
4. Incubate the culture at 30°C, take a 100-μL sample at different time-points (0, 1, 2, 3, 5, 7, 10 h) and plate onto YEA plates.
5. Incubate at 27°C for 72 h.
6. Count the colonies, and calculate the percent survival by comparing with the time-zero control plate.

3.4.2. UV Survival Curves (*see Notes 7 and 8*)

1. Follow **steps 1 and 2 of Subheading 3.4.1.**
2. Plate 100-μL aliquots of the diluted cell culture onto each of 16 YEA plates (500 cells/plate).
3. UV-irradiate the plates using the following doses: 0, 25, 50, 100, 150, 200, 250, and 300 J/m². All UV treatments should be done in duplicate.
4. Incubate the plates at 27°C for 72 h.
5. Count the number of colonies, and calculate the percent survival by comparing with the nonirradiated control plates.

3.5. Microscopic Analysis

The morphology of the mutant cells and their nuclei after exposure to HU can be studied using the DNA-specific fluorescent dye DAPI and an additional dye, calcofluor, that stains material of the septum. The cells are then examined by fluorescence microscopy to determine their morphology.

1. Take cells from an exponentially growing culture, and incubate in YEP containing 20 mM HU at 30°C.

2. Take 100- μ L samples of cells at 2, 6, and 18/24 h.
3. Collect the cells by centrifugation, wash once in water, resuspend in 10 μ L water, and fix in 200 μ L of methanol.
4. Spot 10 μ L of the fixed sample onto a glass slide, and air-dry for 5 min.
5. Pipet onto a cover slip 5 μ L of a water, containing DAPI stain (0.1 μ g/mL) and calcofluor (0.5 μ g/mL), and gently press against the dried fixed cells on the glass slide.
6. Examine the cells using a fluorescent microscope, and determine the percentage of each phenotype (cut and elongated) for each sample (see **Note 9**).

4. Notes

1. Alternative mutagenesis protocol using UV radiation:
 - a. Prepare a fresh culture of log phase cells (as described in **step 1, Subheading 3.1**)
 - b. Plate enough cells onto YEA plates to give ~500 cells per plate surviving mutagenesis (1000-2000 cells per plate assuming a survival rate close to 25–50%).
 - c. Make sure the surface of the plate is well dried, remove the lid and UV irradiate. The UV dose for wild-type cells is ~300 J/m².
 - d. Incubate the cells as described in **step 5 of Subheading 3.1**.
2. Since the most obvious screens are already very close to saturation, any attempt to isolate new genes involved in the DNA structure checkpoint response should be designed with great care, and specific objectives and different targets decided in order to avoid isolating previously cloned genes. For example, a *cdc17* (DNA ligase) mutant can be used in a screen comprising synthetic lethality following a transient shift to the restrictive temperature, or a 48-h incubation at the semipermissive temperature. The DNA ligase thermosensitive mutant when incubated at the restrictive temperature (35.5°C) is defective in the ligation of Okazaki fragments during replication. At the restrictive temperature, the *cdc17* mutant arrests in S-phase, elongates, and slowly loses viability. This late S-phase arrest is distinct from early S-phase arrest caused by HU. Mutations abolishing the S-M checkpoint in a *cdc17* background will make the double mutants highly sensitive to elevated temperatures. Double mutants will rapidly become nonviable after a brief incubation at the restrictive temperature (“transient temperature sensitivity”) or a long incubation at the semipermissive temperature, since they will enter an abortive mitotic event with unreplicated DNA, displaying a cut phenotype. In some aspects, screens using the *cdc17* genetic background mimic the HU mutant screen, but subtle differences exist that may be useful for the isolation of new checkpoint mutants.
 - a. Replica plate the mutagenized *cdc17* colonies onto two plates (one YEA and one YEA + P) as described in **Subheading 3.2**.
 - b. Incubate the YEA master plate at 27°C for 48 h, and the YEA + P plate first at 35.5°C for 9 h and then at 27°C for 48 h, or incubate the YEA master plate at 27°C for 48 h and the YEA + P plate at 31.5°C (semipermissive temperature) for 48 h.

- c. Identify those dead colonies on the YEA + P plate comprised of cells with a cut phenotype. Isolate the corresponding cells from the YEA master plate, and patch onto a new YEA plate.
- d. Confirm the phenotype of these patches by replica plating again onto YEA and YEA + P, repeating step b.
- e. Discard those mutants that do not show a reproducible phenotype.

The 9-h incubation of the *cdc17* mutant at 35.5°C (or 48 h at the semi-permissive temperature of 31.5°C) reduces the viability of the single mutant, but colonies still form. A double mutant composed of *cdc17* and any S-M checkpoint mutant will be nonviable and incapable of forming colonies under these conditions. The use of DNA replication mutants like *cdc20* (DNA polymerase ϵ) that arrest in early S-phase (*cdc17* arrests in late S-phase), is also a potentially useful approach since it may uncover different aspects of the S-M checkpoint pathway.

3. Genetic analysis of checkpoint mutants: To ensure that the phenotype seen in each mutant is the outcome of a single gene mutation and not the result of the interaction between two different genetic mutations, it is essential to backcross each mutant three times with wild-type cells. If after this process the phenotype is retained, it is reasonable to assume that only one gene is responsible for it. In addition these backcrosses have the important effect of ensuring a clean genetic background.
4. Most mutant screens target particular genes preferentially in such a way that many of the generated mutants may be identical (e.g., *rad3* mutants constitute up to 50% of the S-M and DNA damage checkpoint mutants isolated to date). To avoid unnecessary duplication of work by characterization of two identical checkpoint mutants, it is recommended that mutants be crossed to one another and to known checkpoint mutants with similar phenotypes. If the two strains used in a given cross are allelic, then wild-type cells will not be generated from this cross. Note, that if two different genes are closely linked, wild-type cells may be absent or rare. However, linkage between two different nonallelic mutants with a similar phenotype is very rare.
5. Alternative procedure: The use of γ -rays in the isolation of DNA damage checkpoint mutants will primarily isolate mutants deficient in G2-M arrest, since this transition is the most critical in cells exposed to ionizing radiation. The experimental procedure is essentially the same as the one described in **Subheading 3.3.** for the isolation of UV-sensitive checkpoint mutants. A γ -ray dose of approximately 1000–1500 Gy is required.
6. An alternative HU survival test; the spot test.
 - a. Determine the cell density of an exponentially growing culture using a hemocytometer.
 - b. Dilute each culture to four different concentrations (10^7 , 10^6 , 10^5 , and 10^4 cells/mL) in rich medium.
 - c. Make three YEA + P plates containing the following concentrations of HU: 3, 5, and 7.5 mM.

- d. Spot 2 μL of each diluted strain onto YEA + P plates containing the different concentrations of HU so that an increased dilution of the same strain is spotted across the plate. Different cell strains should be spotted in parallel lines on the same plate, so that comparisons of their HU sensitivity can be made.
- e. Incubate the plates at 27°C for 72 h.
- f. Compare the levels of growth.

The spot test and the HU survival analysis described in **Subheading 3.4.1.** may give different results. This is because the spot tests measures an adaptive response to low concentrations of HU, whereas the survival curves measure a survival response to acute exposure to high concentrations of HU.

7. Alternative procedure: γ -ray survival curves. The experimental procedure is similar to the one described in **Subheading 3.4.2.** for testing survival to UV radiation. The plates should be irradiated at the following doses: 0, 50, 100, 200, 400, 500, 1000, and 1500 Gy. If the γ -ray source has a small irradiation chamber the cells should be diluted to the correct cell density (5000 cells/mL), irradiated and only then plated (as described in **Subheading 3.4.2.**).
8. Alternative procedure: EMS survival curves. The experimental procedure is similar to the one described in **Subheading 3.4.1.** for HU survival curves. Incubate the cells in medium containing 2% (v/v) EMS, and take samples as described for determining HU survival.
9. Most DNA damage checkpoint mutants become sensitive to HU at high concentrations or after long incubations, but under standard treatment conditions, they have a normal checkpoint response and are not particularly sensitive to HU. Non-checkpoint DNA repair mutants, when incubated with DNA-damaging agents die with a highly elongated phenotype, because they are unable to repair the DNA damage. Some extremely sensitive DNA repair mutants die with no elongation at normal doses of mutagens. This is because they cannot undertake transcription. At very low concentrations of DNA-damaging agents, such mutants will display a highly elongated phenotype.

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Isolating Mutants of the Nematode *Caenorhabditis elegans* That Are Hypersensitive to DNA-Damaging Agents

Phil S. Hartman and Naoaki Ishii

1. Introduction

The nematode *Caenorhabditis elegans* has gained widespread popularity for use in addressing many biological problems, particularly those relating to development (for brief topical reviews, *see 1–5*; for comprehensive treatises, *see 6–10*). This can be attributed to both inherent properties of the organism as well as the collegiality extant within the “worm community.” With respect to the former, *C. elegans* is extremely easy to grow in the laboratory (animals are typically propagated on agar-filled Petri dishes seeded with the bacterium *Escherichia coli*) and possesses a short generation time (3 d at 20°C). The system is genetically robust, with the availability of thousands of mutants as well as the existence of a physical map whose sequencing (over 82 Mb finished at present) is scheduled for completion in 1999. Developmental studies have been advantaged by the animal’s transparent nature, facilitating complete elucidation of *C. elegans*’ largely invariant cell lineage.

The collegiality of the worm community is manifested as follows:

1. There is a *Caenorhabditis* Genetics Center (University of Minnesota; E-mail stier@biosci.cbs.umn.edu) that maintains many stocks and freely disseminates them on request.
2. Investigators frequently exchange information prior to publication via the informal *The Worm Breeder’s Gazette* (E-mail stier@biosci.cbs.umn.edu for subscription particulars).
3. An electronic news group exists for discussion and announcements related to *C. elegans* (to subscribe by E-mail, send the message “subscribe CELEGANS” to

biosci-server@net.bio.net), allowing individuals to share information readily as well as solicit widespread input to queries.

4. Several Web sites may be accessed (e.g., elegans.swmed.edu, www.dartmouth.edu/artsci/bio/ambros/protocols.html, probe.nalusda.gov:8000/acedocs/allace.html) in order to obtain protocols, various literature, and sequence information.

With respect to the processing and consequences of DNA damage in *C. elegans*, several areas have received particular attention (reviewed in **I**). These include the developmental regulation of DNA repair, the lethal and mutagenic effects of cosmic radiation (as it relates to long-term human travel in space), and the effects of DNA damage on cellular and organismal aging.

Two protocols for isolating mutants of *C. elegans* hypersensitive to DNA-damaging agents are described below. The first protocol is analogous to the replica-plating methodology developed by the Lederbergs (**II**), although it is considerably more labor-intensive (cf. Chapter 6). It was developed by Hartman and Herman (**12**), who screened over 6400 clones to isolate nine radiation-sensitive (*rad*) mutants. In brief, individual second-generation progeny (F_2 s) of mutagenized animals are placed in a first set of separate wells ("rescue wells") of a microtiter plate and allowed to reproduce. They are then transferred to fresh wells ("treatment wells") and insulted with a DNA-damaging agent under conditions sublethal to wild type. Several days after transfer, the second set of wells is examined; in them, candidates will have produced either very few offspring or a preponderance of progeny arrested at embryonic or early larval stages. Candidates can then be propagated and retested using animals from the rescue wells.

The second protocol, termed "embryo rescue," is peculiar to *C. elegans* and has as a primary advantage the fact that "replica plating" is not necessary. It is made possible because the "eggshell" of developing embryos is impervious to most chemicals, including many DNA-damaging agents. Thus, exposure to the toxic chemical may kill the mutant itself, but its *in utero* progeny will survive, allowing propagation of putative mutants. In this procedure, the F_2 s of mutagenized animals are incubated for several hours in a solution containing a relatively high drug concentration. They are then plated on Petri dishes en masse. Wild-type animals survive this treatment and begin movement within minutes after plating. Conversely, drug-sensitive mutants die and are therefore immobilized. The latter are plated on individual drug-free Petri dishes and their progeny retested. This protocol has been employed successfully by one of us to isolate two methyl viologen-sensitive mutants, *mev-1* and *mev-2*, from about 15,000 F_2 progeny of ethyl methanesulfonate- (EMS) treated animals (**13**).

2. Materials

The following reflect the materials specifically employed in the authors' laboratories. More varied and extensive descriptions of the materials necessary

to propagate *C. elegans* are readily available (7,8; www.dartmouth.edu/artsci/bio/ambros/protocols.html).

1. Wild-type *C. elegans*, strain N2 (*Caenorhabditis* Genetics Center, University of Minnesota).
2. Dissecting microscope: Animals can be readily observed with transmitted light at 20× magnification.
3. Although animals can be grown in liquid, the methods described below employ a solid medium such as MYOB: 5.9 g of stock, 20 g of agar/L of H₂O. Stock is: 55 g Tris-HCl, 24 g Tris-OH, 310 g Bacto-peptone, 800 mg cholesterol, 200 g NaCl. This medium should be autoclaved, cooled to 55°C, and either poured into 35-, 60-, or 100 mm Petri dishes or pipeted into 24-well microtiter dishes (see **Notes 1 and 2**). After the medium has solidified, the surface is inoculated with an overnight culture of *E. coli* OP50, a uracil auxotroph (available from the *Caenorhabditis* Genetics Center, University of Minnesota), and incubated at 20°C for at least 12 h before inoculation with nematodes. A single drop is sufficient bacterial inoculum and can be spread with a sterile glass spreader on Petri dishes.
4. M9 buffer: 5.8 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl/L of H₂O.
5. 32-Gauge platinum wire (ca. 1.5 cm in length) affixed to a handle (e.g., one designed for bacterial inoculations) is used to transfer individual animals. The wire should be flame-sterilized before a transfer is effected. With some practice, individual or small groups of animals (visualized under the microscope) can be scooped off the surface and transferred to another. Care should be taken not to gouge the agar's surface, since the animals will burrow. Typically, two to three animals are transferred from one plate to another for stock maintenance. Most strains of *C. elegans* will starve the bacterial lawn within 1 wk of transfer, although stocks need to be transferred only once every several weeks. Animals are typically grown at 20°C, with 15° and 25°C the permissive and restrictive temperatures for temperature-sensitive mutants.

3. Methods

3.1. Replica Plating

Although a number of mutagens have been employed successfully with *C. elegans* (reviewed in **14**), EMS is most commonly used. The following is a modification of the protocol recently reviewed by Anderson (**14**).

1. To obtain semisynchronous cultures of wild-type nematodes for EMS treatment, starve the plates of bacteria for between 1 and 2 wk before usage. Such plates will contain a few geriatric adults and many young (L1 and L2) larvae.
2. Wash these off the plate in M9 buffer, and pellet in a clinical centrifuge.
3. Using a micropipet or Pasteur pipet, inoculate the pellet of worms onto a 100-mm Petri dish seeded with bacteria. The nematode inoculum should be small enough (ca. 100–500) such that the bacterial lawn does not become starved. Incubate for 2 d.

4. Wash the plate (now containing primarily fourth-stage [L4] larvae and young adults) with 2 mL of M9 buffer, and add the contents to 2 mL of M9 buffer to which 10 μ L of EMS was previously dissolved. After 4 h at 20°C, wash the worms once in M9, and plate on seeded 100-mm Petri dishes at a density of ~10–20/plate. Incubate at 20°C for 5–7 d.
5. Transfer individual second-generation (F_2) L4s to individual agar-filled wells of 24-well microtiter plates, which serve as “rescue” wells. Such animals should be abundant between d 5 and 7 after mutagenesis.
6. After 24 h, transfer the F_2 s (now egg-laying adults) to a second “treatment” well and immediately expose to a DNA-damaging agent. Both 254 nm UV radiation and methyl methanesulfonate (MMS) have been employed successfully in such mutant hunts. UV radiation can be imposed (with the lids off !) by a single germicidal fluorescent 15-W bulb that produces approx 1 J/m²/s at a distance of 55 cm. MMS should be added to the molten agar to achieve a final concentration of 0.1 mM immediately before these wells (but not the rescue wells) are poured.
7. After 72–96 h, examine the treatment wells. The majority will contain nonmutants and will have >50 F_3 s and F_4 s ranging in size from embryos to adults. Putative mutants will be signaled by wells containing either very few animals or a preponderance of animals arrested at embryonic or early larval stages. Most of these are not hypersensitive to the DNA-damaging agent. Instead, they contain a mutation in some essential gene unrelated to DNA damage tolerance. These are evident from inspection of the rescue wells, which will contain a similar distribution of animals as in treatment wells. Only those clones with robust growth in the rescue well, but impaired growth in the treatment well are worthy of retesting.
8. Candidates should be retested as above. With EMS-mutagenized populations, approx 1% of the clones will pass the first screening. Of these candidates, over 80% will prove to be false-positives (see **Note 3**).

3.2. Embryo Rescue

1. Treat a population of wild-type animals with EMS as described in **steps 1–4 of Subheading 3.1**.
2. Seven days after mutagenesis, wash the F_2 animals off the Petri dishes with M9 buffer, and incubate in 30 mM methyl viologen (or some other chemical DNA-damaging agent) for 4 h at 20°C.
3. After this treatment, wash the animals free of methyl viologen and spot in the middle of a 100-mm Petri dish containing a lawn of *E. coli*.
4. Twenty-four hours after plating, most animals will have recovered and crawled away from the center of the plate. Transfer the carcasses of dead animals at the center of the plate onto individual 35-mm or 60-mm Petri dishes containing an *E. coli* lawn on MYOB.
5. After 3 d, the *in utero* embryos (resistant to the chemical by virtue of their impermeous eggshells) should have developed into gravid adults. Retest several from

each plate by transferring them to seeded MYOB plates impregnated with 0.2 mM methyl viologen.

6. Inspect these dishes after 3–4 d. Most wild-type embryos will have developed into L4 larvae or adults. Conversely, *mev* mutants will have arrested as L1 or L2 larvae (see **Note 3**).

4. Notes

1. Although the microtiter dishes are “disposable,” they can be reused. To do so, the plugs of agar should first be removed with a spatula before they desiccate. The dishes should then be washed several times in warm, soapy water, soaked overnight in 1% sodium hypochlorite (common bleach diluted 1:5), and rinsed several times with deionized water. The wells can then be refilled with agar. If bacterial or fungal contamination become problematic, the microtiter dishes can be exposed to UV light for several hours before the agar is poured. In this event, the lids should be removed, and the light source positioned such that, as much as possible, the sides of the wells are exposed directly to the light (UV is poorly penetrant through plastic).
2. It is important that no condensation is present in the microtiter plates, since nematodes may crawl from well to well. It is for this reason that protocols employing either 96-well microtiter dishes or liquid culture have not proven successful in our hands.
3. Once mutants are isolated, they may be analyzed as explained in ref. (12). In addition, as with other mutants in *C. elegans*, the genes may be cloned by transformation rescue (15) once they have been mapped reasonably precisely. Owing to the alignment of the genetic and physical maps in *C. elegans*, precise mapping allows the investigator to employ YACs and cosmids corresponding to the genetically defined region. In addition, knowledge of the DNA sequence gained from the sequencing project can provide the investigator hints concerning specific DNA sequences that may encode the gene.

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Isolating DNA Repair Mutants of *Drosophila melanogaster*

Daryl S. Henderson

1. Introduction

The fruitfly *Drosophila melanogaster* offers numerous advantages as a metazoan model for genetic dissection of conserved biological processes, such as DNA repair. Its ease of culture, short generation time, small number of linkage groups ($2n = 8$), and giant polytene chromosomes, combined with a wealth of morphological mutants and chromosomal variants (*1*) accumulated over 90 years and now cataloged in FlyBase (<http://flybase.bio.indiana.edu> or <http://www.ebi.ac.uk/flybase/>), make it a powerful and versatile system for genetic analysis (*2*). *D. melanogaster* also has emerged as one of the best multicellular eukaryotes in which to disrupt genes by transposon mutagenesis for the purpose of molecular cloning (*3–5*), and to study cloned gene functions by transformation (*6*). Cytological studies of flies also have reached new levels of sophistication in keeping with recent advances in microscopy, probe technology, and electronic imaging (*7,8*).

The use of *Drosophila* in mutagenesis research dates back more than 70 years to H. J. Muller's momentous discovery of the mutagenic action of X-rays (*9,10*). His work, together with that of L. J. Stadler on maize (*11*), effectively ushered in the field of radiation genetics. In the 1940s, Auerbach and Robson used *Drosophila* to demonstrate unequivocally that chemicals, too, can have mutagenic effects (*12,13*). An historical account of their work and of mutation research in general can be found in ref. (*14*). Beginning in the early 1970s, the scope of mutation research in *Drosophila* was broadened by the isolation of mutants potentially deficient in DNA repair. Such mutagen-sensitive (*mus*) mutations render embryos and larvae hypersensitive to the lethal effects of DNA-damaging agents. The first *mus* mutations were recovered on the X chro-

Table 1
Cloned Mutagen-Sensitive Genes

<i>Drosophila</i> gene ^a	Homolog ^b	Reference
<i>mei-9</i>	<i>XPF</i>	(22)
<i>mei-41</i>	<i>ATM</i>	(23,24)
<i>mus101</i>	<i>rad4/cut5</i> <i>S. pombe</i>	R. M. Raupp, J. M. Axton, D. M. Glover, and D. S. Henderson (unpublished results)
<i>mus205</i>	<i>REV3</i> <i>S. cerevisiae</i>	(25)
<i>mus209</i>	<i>PCNA</i>	(26)
<i>mus308</i>	?	(27)
	(Novel protein with putative helicase and polymerase domains)	
<i>mus309</i>	<i>Ku70</i>	(28)

^aX-linked *mus* mutants are numbered beginning with 101, 102, and so forth. Those on chromosome 2 are numbered 201, 202, and so on for chromosome 3. Two X-linked mutagen-sensitive loci, *mei-9* and *mei-41*, are exceptions. They carry the designation *mei*, for *meiotic*, instead of *mus* because the first mutant alleles of these genes were recovered in screens for meiotic abnormalities (29), and later found to be mutagen-sensitive (16,17,30). With the exception of *mus308*, mutants in all of these genes were isolated on the basis of sensitivity to MMS; *mus308* mutants are preferentially sensitive to crosslinking agents (e.g., HN2).

^bHuman homolog except where indicated.

mosome in screens that employed methyl methanesulfonate (MMS) as a selective agent (15–17). Subsequent screens using a variety of mutagens identified *mus* mutations on chromosomes 2 and 3 (18–20) and brought to more than 30 the number of *mus* genes documented in *Drosophila* (1). The notion that *mus* mutations disrupt DNA repair-related genes has since been confirmed by biochemical assays (21) and more recently by molecular cloning (see Table 1).

Screens for mutants in *Drosophila* usually target a specific chromosome—either the X, the 2nd, or the 3rd—unlike those in other organisms, which typically screen entire genomes at a time (see Chapters 1, 2, 4–6). Such specificity is possible with flies because of the existence of balancer chromosomes. Balancer chromosomes carry multiple inversions that suppress meiotic recombination and dominant genetic markers that allow them to be traced through successive generations. The two major autosomes, chromosomes 2 and 3, each constitutes ~40% of the euchromatic part of the genome, whereas the X chromosome makes up nearly all of the remaining ~20%. Chromosome 4 is so small, accounting for only ~1% of the euchromatic genes, that systematic screens for 4th chromosome mutants have not been considered worth the effort.

In general, X chromosomal screens are faster and easier than autosomal screens, a point made clear in **Subheading 3.2.**

This chapter describes strategies for isolating *mus* mutants on the X and 2nd chromosomes. Chromosome 3 *mus* mutants can be isolated by introducing a simple modification to the scheme used to isolate mutants on chromosome 2. In addition, both screens are designed to permit identification of temperature-sensitive (ts) mutants. **Subheading 3.1.** describes how to induce germline mutations by feeding ethyl methanesulfonate (EMS) to adult males. Males thus treated are mated to appropriately genetically marked strains to establish, after a series of crosses, a collection of lines in which each line potentially carries a unique EMS-induced mutation on the X or 2nd chromosome (**Subheading 3.2.**). Larvae from each line are then tested for hypersensitivity to one or more DNA-damaging agents. These tests are done in such a way that each mutagen-treated culture contains potentially mutagen-sensitive larvae together with mutagen-insensitive (*mus*⁺) siblings, the latter serving as an internal control. Since these two classes of flies are readily distinguishable from one another by their phenotypic markers (see **Subheading 3.2.**), absence of the first class in any mutagen-treated culture indicates a putative mutagen-sensitive strain. Putative mutants are then retrieved from a stock collection for retesting and further characterization.

2. Materials

2.1. Fly Strains

1. Isogenic line (see **Note 1**) carrying a visible marker (or markers) appropriate for the chromosome to be screened: e.g., *w* (*white*) for the X chromosome; *b pr cn* (*black body, purple eyes, cinnabar eyes*) for chromosome 2; *st* (*scarlet eyes*) or *red e* (*red Malpighian tubules, ebony body*) for chromosome 3 (see **Note 2**). Before undertaking a screen, the line should be tested to ensure it is not already hypersensitive to mutagens.
2. Attached-X stock, e.g., *C(1)DX,y f/Y* (referred to here as X[^]X/Y), for screening the X chromosome. Balancer chromosome stock, e.g., *Gla/CyO* for screening chromosome 2 or *Ly/TM3,Sb* for screening chromosome 3. (See **Note 3**.)

2.2. Mutagenesis

1. 25 mM EMS (e.g., Sigma, St. Louis, MO) in 1% v/v aqueous sucrose. Prepare fresh. EMS is listed as methanesulfonic acid ethyl ester in Sigma's catalog. The density of EMS is 1.17 g/mL. Store the bottle wrapped in parafilm at 4°C. Handle with gloves in a fume hood.
2. Denaturing solution: 1 M NaOH, 0.5% v/v thioglycolic acid. Prepare fresh.
3. Glass or plastic fly culture bottles.
4. Whatman paper filters (e.g., no. 4) cut as circles to fit tightly inside the bottom of the bottle.
5. 2-mL Syringe with long needle (e.g., 21 gage, 1½ inches).

2.3. Mutant Screen

1. Basic equipment for *Drosophila* culture, such as vials, bottles, anesthetizers, dissecting microscope, and so forth, is required (**31**).
2. Mutagens (*see* **Note 4**): Nitrogen mustard (HN₂; mechlorethamine hydrochloride; Sigma). To make a 1% (w/v) stock solution, dissolve 1 g of HN₂ (i.e., the entire contents of a bottle) into several milliliters of 0.1 N HCl, and dilute to a final volume of 100 mL. Dispense into 1-mL aliquots, and store at -20°C. Handle with gloves in a fume hood. Decontaminate all pipets, glassware, and so forth, and destroy any unwanted HN₂ with denaturing solution (*see* **item 2, Subheading 2.2.**). MMS (e.g., Sigma). MMS is listed as methanesulfonic acid methyl ester in Sigma's catalog. Store the bottle of MMS wrapped in parafilm at 4°C. Handle with gloves in a fume hood.
3. Denaturing solution: *see* **item 2, Subheading 2.2.**
4. Multipipeter (e.g., Eppendorf Multipette® and 12.5 mL Combitips, Brinkmann Instruments, Westbury, NY).
5. Large incubators set at 22°C and 29°C (if screening for *ts mus* mutants).

3. Methods

3.1. EMS Mutagenesis

For historical reasons and because it is effective and relatively nontoxic to the adult fly, EMS is the most commonly used chemical for inducing germline mutations in *Drosophila*. Alternative mutagenesis procedures may be worth considering (*see* **Notes 5 and 6**).

1. Place ~100 males (e.g., *w* or *b pr cn*) into each of several bottles containing two layers of filter paper fitted tightly at the bottom of the bottle. Leave the flies to starve overnight.
2. The next day, prepare 0.5–1 L of EMS denaturing solution. This should be used to decontaminate all pipet tips, glassware, and so forth, and any spills.
3. Prepare a 25-mM EMS/sucrose solution as follows. In a fume hood and wearing gloves, add 66 µL of EMS to 25 mL of a 1% sucrose solution. The EMS will not go into solution right away, but will sink as droplets to the bottom of the beaker. These droplets should be dispersed by drawing them up, along with several milliliters of sucrose solution, into a 2-mL syringe and expelling the solution back into the beaker. Repeat several times until all the EMS is in solution. To ensure a homogeneous solution, mix using a stir bar and magnetic stirrer. (*See* **Note 7.**)
4. Using the 2-mL syringe, dispense 1–1.5 mL of EMS/sucrose solution into each bottle containing flies. Insert the needle through the bottle stopper (or cotton plug) taking care not to let any flies escape, and dampen the filter paper with EMS solution. Avoid soaking the filter paper, since this may cause the flies to stick. Allow the flies to imbibe overnight in the fume hood.
5. Decontaminate the pipet tip, syringe, and any remaining EMS solution with denaturing solution. Use 1 vol of denaturing solution/1 vol of EMS solution. Leave in the fume hood for 1–2 d before discarding.

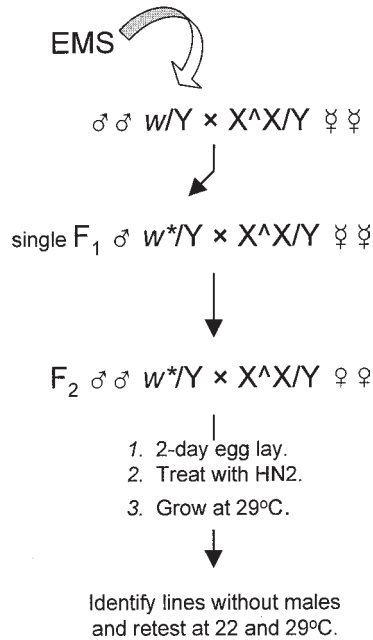


Fig. 1. Diagram of the crossing scheme used to isolate X-linked *mus* mutations. See **Subheading 3.2.1.** for details. The females in these crosses produce two types of gametes: X^X -bearing ova and Y -bearing ova. The males produce both X - and Y -bearing sperm as usual. The X chromosome that is retained in males in these crosses is denoted simply by its marker, w . Of the four types of zygotes produced by these matings, two are viable (w/Y and X^X/Y) and two are inviable (w/X^X and YY).

6. The next day, transfer the flies to bottles containing food to allow them to recover. Tap the flies to the bottom of the EMS-treatment bottle, quickly remove the stopper, invert the bottle over the new food-containing bottle, and tap gently to transfer the flies. Leave the flies to feed overnight. Pour denaturing solution into the EMS-treatment bottle, and leave for 1–2 d in the fume hood.

3.2. Screen for Mutagen-Sensitive Mutants

3.2.1. X-Linked Mutants (Fig. 1)

1. Cross mutagen-fed w males (from **Subheading 3.1.**) with X^X/Y virgin females (see **Note 8**) in bottles en masse. Use ~2–3 females for every male. Transfer the parents to fresh bottles every 1–3 d, depending on the number of eggs laid and the level of hatching (see **Note 9**). Grow these cultures at 22°C (the permissive temperature). At this time set up bottle stocks of X^X/Y flies so that X^X/Y virgins will be available for collecting at the time the F_1 males eclose.

2. Collect w^*/Y F_1 males (where * indicates an X chromosome potentially carrying an EMS-induced mutation [see **Note 10**]) and cross each one separately to 3–4 X^AX/Y virgin females in vials. Label each vial, e.g., with an alphanumeric code. Grow at 22°C. This step establishes a collection of distinct lines to be tested for mutagen-sensitivity as described in **steps 3–7**. (See **Notes 11** and **12**.)
3. Transfer the F_2 generation of each line to vials containing fresh medium (see **Note 13**), and allow the females to lay eggs for 2 d at 22°C. (See **Note 14**.)
4. At the end of 2 d, transfer the parents to fresh vials. Keep the second set of vials at 22°C as stocks. (See **Note 15**.)
5. Prepare 0.5–1 L of denaturing solution.
6. In a fume hood and wearing gloves, prepare 0.008% w/v HN2 solution (see **Note 4**). To treat approx 400 vials, pipet 800 μ L of 1% HN2 stock solution into a 250-mL beaker containing 100 mL of distilled water. Mix using a stir bar or by pipeting with a 10-mL pipet. Decontaminate all pipets, glassware, and so forth, with denaturing solution and leave them in the fume hood for 1–2 d.
7. In a fume hood, using a multipipeter, dispense 0.25 mL of HN2 solution onto the food surface of each 2-d-old culture (consisting of mostly embryos and a few first instar larvae). Leave the treated cultures in the fume hood for 1 d before transferring them to 29°C (the restrictive temperature) for the remainder of development (~7–10 d; see **Note 16**).
8. Determine the male:female ratio in each vial (F_3 generation). Be sure that any late eclosing flies are counted. Vials with no or very few males (see **Note 17**) and significant numbers of females contain putative mutants belonging to one of the following classes: ts lethal mutants (the majority caused by mutations in essential genes having nothing to do with DNA repair), ts *mus* mutants, non-ts *mus* mutants, or false positives. Retrieve all such lines from the stock cultures for retesting.
9. Retest the putative mutants using the protocol described in **steps 5–7**, except that for each line, set up cultures at 22 and 29°C, both with and without mutagen treatment. Approximately 6–10 male-female pairs/vial (depending on fecundity) should be sufficient for these retests. Allow the females to lay eggs for 2 d, and then transfer the parents to new vials to establish a second set of cultures or “replicas.” Treat the first cultures with mutagen, and use the replicates as untreated controls. Use at least 3 vials/line for these retests. (see **Notes 18** and **19**.)
10. Determine the male:female ratio in each vial. **Table 2** summarizes the phenotypic classes that can be expected.
11. Each confirmed *mus* mutant should be characterized further, e.g., by mapping the mutation, testing for allelism with other *mus* mutants, testing for sensitivity to other DNA-damaging agents, generating dose–response curves, and so forth (**16,17,32**).

3.2.2. Autosomal Mutants (**Fig. 2**)

The following protocol describes the steps necessary to isolate *mus* mutants on chromosome 2. A similar procedure can be followed to isolate mutants on

Table 2
Classification of Mutants^a

Mutant category	22°C		29°C	
	+ Mutagen	– Mutagen	+ Mutagen	– Mutagen
<i>mus</i> (non-ts)	L	V	L	V
<i>mus</i> ^{ts}	V	V	L	V
<i>mus</i> ^{tsl}	L	V	L	L
ts lethal	V	V	L	L

^aL = lethal; V = viable. Lines exhibiting a *mus*^{tsl} phenotype must be characterized further (e.g., by mapping) to determine if the two phenotypes (mutagen sensitivity and ts lethality) are caused by the same mutation. Mutants of this type have been identified in *Drosophila*, e.g., *mus209^{Bl}* (26).

chromosome 3, except that 3rd chromosome markers and balancer chromosomes must be used.

1. Cross mutagen-fed *b pr cn* males (from **Subheading 3.1.**) to *Gla/CyO* virgin females in bottles en masse. Grow at room temperature. (See **Note 20.**) Transfer the parents to fresh bottles every 1-3 d depending on the number of eggs laid and the level of fecundity (see **Note 9**).
2. Collect *b pr cn*/CyO* and *b pr cn*/Gla* F₁ males, and mate each one separately to 3-4 *Gla/CyO* virgin females in vials, where *b pr cn** represents a 2nd chromosome potentially carrying an EMS-induced mutation (see **Note 21**). Label each vial, e.g., using an alphanumeric code, to keep track of each line.
3. From each vial collect *b pr cn*/ CyO* male and virgin female F₂ siblings and allow them to mate in a vial containing fresh medium. (Discard all *Gla*-bearing F₂ flies.) Grow at 22°C.
4. Check the F₃ progeny for the presence of *b pr cn* homozygotes. These are readily distinguishable from their *b pr cn/CyO* siblings; the former have black bodies and straight wings, but the latter have wild-type body color and curly (Cy) wings. An absence of *b pr cn* homozygotes in any culture containing significant numbers of Cy F₃ flies indicates the presence of an induced recessive lethal mutation. Such lines should be discarded (see **Notes 22 and 23**).
5. Transfer the remaining lines to fresh vials (see **Note 13**), and allow the females to lay eggs for 2 d.
6. At the end of 2 d, transfer the parents to new vials, and keep as stocks at 22°C. (See **Note 15**.)
7. Prepare 0.5–1 L of denaturing solution.
8. In a fume hood and wearing gloves, prepare 0.06% (v/v) MMS solution. To treat approx 400 vials, pipet 60 µL of MMS into a 250-mL beaker containing 100 mL of distilled water. As with EMS, MMS will form droplets at the bottom of the beaker. Disperse these droplets using a needle and syringe as described for EMS (see **step 3, Subheading 3.1.**).

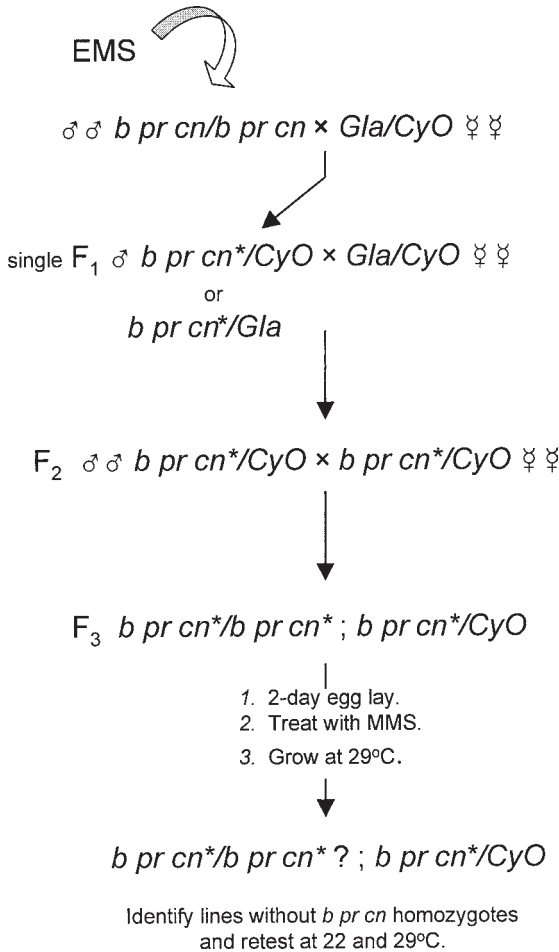


Fig. 2. Diagram of the crossing scheme used to isolate *mus* mutations on chromosome 2. See **Subheading 3.2.2.** for details.

9. In a fume hood, using a multipipeter, dispense 0.25 mL of MMS solution onto the food surface of each 2-d-old culture (consisting of mostly embryos and a few first instar larvae). Leave the treated cultures in the fume hood for 1 d before transferring them to 29°C (the restrictive temperature) for the remainder of development (~7–10 d; see **Note 16**).
10. Count the numbers of *b pr cn* homozygotes and *b pr cn/CyO* heterozygotes in each mutagen-treated vial. Lines having no or significantly reduced numbers of homozygotes are putative mutants belonging to one of the following classes: ts lethal mutants (the majority caused by mutations in genes unrelated to DNA repair), ts *mus* mutants, non-ts *mus* mutants, or false positives. (See **Note 24**.)

Retrieve all such lines from the stock cultures for retesting.

11. Retest the putative mutants as described in **step 9** of **Subheading 3.2.1. Table 2** lists the categories of mutants that can be expected.
12. Confirmed *mus* mutants should be characterized further as outlined in **step 11, Subheading 3.2.1. (18–20)**.

4. Notes

1. All individuals in an isogenic line carry identical copies of a particular chromosome derived by descent. For example, to make a stock isogenic for the X chromosome, cross a single *w* male to several X^+X/Y females, and expand this line in bottles. All male descendants will carry identical X chromosomes. To make a stock isogenic for chromosome 2, cross a single *b pr cn/CyO* male to several *Gla/CyO* females. Mate the *b pr cn/CyO* F₁ siblings. Mate the *b pr cn* homozygous F₂ siblings to establish an isogenic *b pr cn* stock. Isogenic stocks are free of recessive lethal mutations, which may be segregating in nonisogenic lines. Over time, however, isogenic lines will become nonisogenic as they accumulate random mutations.
2. Do not use *ry* (*rosy*) as a third chromosome marker. *ry* mutants, which are defective in xanthine dehydrogenase, are hypersensitive to killing by oxygen radical-generating agents, such as ionizing radiation and paraquat (methyl viologen), apparently because they are unable to synthesize the antioxidant uric acid (33).
3. Balancer chromosomes (1,2), such as *CyO* (“curly-Oh”) carry multiple inversions that suppress crossingover in females (there is normally no meiotic recombination in *Drosophila* males). *CyO* carries the dominant marker *Curly* (*Cy*) wings (and several recessive visible markers, including *pr* and *cn*), which allows its segregation to be followed from one generation to the next. *Gla* (*Glazed*) is a dominant mutation that gives the eyes a smooth, shiny appearance (1).
4. In principle, any mutagenic agent can be used for screening. However, for some chemicals, finding a suitable (nontoxic) solvent can be problematic, and so too detoxification and disposal. MMS has been used to great effect, but in the case of the X chromosome, it is unlikely that many new MMS-sensitive loci will be identified. Certainly any such screen using MMS will encounter diminishing returns. Rather, the use of other agents, such as crosslinking compounds (e.g., nitrogen mustard), might yield mutants in new X-linked *mus* genes. Autosomal screens do not yet have this limitation.
5. Ethyl nitrosourea (ENU) can be used as an alternative mutagen for adult feeding. ENU has a greater propensity than EMS to alkylate O⁶-guanine, and may therefore produce a different spectrum of mutations (34). Like EMS, ENU is highly genotoxic and should be handled with extreme care using gloves, and so on, with all operations carried out in a fume hood. Prior to working with ENU, prepare 1 L of 1 M NaOH to be used to decontaminate all equipment. The following procedure is modified from Ashburner (35). To avoid the potential hazards associated with weighing out ENU on a balance, the use of an ISOPAC® (Sigma) is recommended. These are available for a number of different mutagens/carcinogens and contain a preweighed amount (approx 1 g) of solid chemical in a serum bottle

sealed with a butyl rubber stopper. (ENU is listed as *N*-nitroso-*N*-ethylurea in the Sigma catalog.) Using a 5 or 10 mL syringe and ~18-gage needle, inject a total of 100 mL of 10 mM acetic acid to give a 1% ENU stock solution. Before injecting the acetic acid solution, insert a second 18-gage (or smaller) needle to help vent the displaced air. After the ENU is dissolved, dispense into 1-mL aliquots and store at -70°C . (A disadvantage of ISOPACs is that you end up with far more mutagen than you may ever require.) Decontaminate all surfaces with household ammonia. For mutagenesis treatment, thaw an aliquot of ENU, and add it to 24 mL of 1% aqueous sucrose solution to give a final concentration of 0.4 mg/mL (3.4 mM). Feed to adult males as described for EMS. Immediately decontaminate all glassware, pipet tips, syringes, and so forth, with 1 M NaOH before disposing of according to local regulations.

6. An attractive alternative to inducing mutations with chemicals is to use P element transposon mutagenesis such as described in refs. (3–5). This has the advantage of allowing P-tagged genes to be cloned with relative ease by the so-called plasmid rescue technique (36). However, P element mutagenesis effectively precludes the isolation of ts mutants and reduces the chance of recovering viable mutants at those *mus* loci that also have essential functions.
7. Alternatively, add the EMS to 25 mL of sucrose solution in a disposable 50-mL polypropylene centrifuge tube and vortex.
8. In *Drosophila*, sex is determined not by the presence or absence of a Y chromosome, as it is in humans, but by the ratio of X chromosomes to sets of autosomes (37): XXY flies are normal fertile females; XO flies are males, but because they lack a Y chromosome, which carries genes indispensable for male fertility, they are sterile.
9. To avoid mutations induced at premeiotic stages, which may result in clusters of the same mutation, cull the male parents after 5–6 d.
10. The paternally derived autosomes will also have been exposed to mutagen. However, these become diluted out in successive generations.
11. X-linked (non-ts) lethals are selected against at this step, since males carrying such mutations are not recovered.
12. In principle, the progeny from the crosses made in **step 2** could be tested for mutagen sensitivity. However, it is more convenient to test the following generation when there are more flies per line to lay greater number of eggs for treatment.
13. Use vials in which food is not splashed on the inside wall. Otherwise, eggs may be laid there and escape mutagen treatment.
14. To stimulate the females to lay sufficient numbers of eggs in 48 h it may be necessary to sprinkle dry yeast pellets onto the surface of the food. Aim for at least ~100 eggs/vial. This should produce ~25 mutagen-insensitive $X^{\wedge}X/Y$ female progeny against which to compare the sensitivity of the males. Half the zygotes produced in this cross ($X^{\wedge}X/X$ and YY) are inviable.
15. Several different mutagenic agents can be tested in succession. To do this, simply transfer the parents every 2 d to generate the required number of replicate cultures.

16. Mutagen treatment causes developmental delay, the length of which varies with the mutagen and the dosage.
17. Keep all lines in which there are, for example, <10% of the expected number of males, assuming an equal number of males and females in the absence of treatment.
18. Higher concentrations of mutagen can be used at 22°C than at 29°C. For example, although a 0.1% concentration of MMS can be used at 22°C, it is toxic even to wild-type at 29°C, possibly because the nearly twofold higher rate of development at that temperature allows less time for repair of potentially genotoxic lesions.
19. If using a mutagen that is dissolved in a solvent other than water, the replicate cultures without mutagen should be treated with the solvent.
20. Alternatively, cross the EMS-treated males to *DTS/CyO* virgin females and grow the F₁ progeny at 28–29°C. *DTS* is a dominant temperature-sensitive lethal mutation (there are several different ones available; consult FlyBase), which kills heterozygotes at 28–29°C. At step 2, cross single *b pr cn*/CyO* males to 3–4 *DTS/CyO* virgin females, and grow at 28–29°C. This eliminates the need to collect virgins at **step 3**. (*CyO* homozygotes are inviable.)
21. The Y, the 3rd, and the 4th chromosomes will also have been treated with mutagen. The Y chromosome is largely heterochromatic, carrying six genes required for male fertility. The 3rd and 4th chromosomes will be randomized in subsequent generations.
22. As much as 50–60% of all lines may carry recessive lethal mutations.
23. It may be that some ts mutants are viable only at temperatures lower than 22°C. Before discarding the lethal lines, it may be worth growing them at 18°C to check for viability. The reason 18°C is not used as the permissive temperature in the actual screen is that it lengthens considerably the generation time, to nearly 1 mo. Alternatively, give the unwanted lines to colleagues who may wish to screen them for other phenotypes.
24. Lines that when treated with a mutagen consistently yield no (or very few) homozygotes and low numbers of *b pr cn/CyO* heterozygotes may be semidominant mutants. To test this possibility, outcross the *mus* mutant to a wild-type line, and collect *mus/+* F₁ males. Cross these back to *mus/CyO* virgin females, and test their progeny for mutagen sensitivity. Compare the survival of *mus/mus*, *mus/+*, *mus/CyO*, and *+/CyO* genotypes.

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Generation, Identification, and Characterization of Repair-Defective Mutants of *Arabidopsis*

Anne Britt and Cai-Zhong Jiang

1. Introduction

UV radiation induces two major DNA damage products: the cyclobutane pyrimidine dimer (CPD) and the pyrimidine[6-4]pyrimidinone dimer (or [6-4] photoproduct; [6-4]PP). The biological effects of both lesions have been studied in microbial and mammalian systems. Pyrimidine dimers have been shown to act as blocks to the progress of microbial and mammalian DNA polymerases and to inhibit DNA replication both in *cis* and in *trans*. These dimers have also been shown to inhibit the progress of mammalian RNA polymerases and, as a result, to eliminate the expression of a transcriptional unit. The direct biological effects of UV-induced pyrimidine dimers on DNA replication and transcription have not been well studied in plants. However, it has been well documented that increasing doses of UV radiation can result in slower plant growth, a generalized stress response, or death of the irradiated tissues. UV irradiation of pollen can induce mutations; presumably dimers play a role in this process, as the mutagenic effects of UV radiation are photoreactivatable.

The isolation and analysis of UV-sensitive mutants is a useful way to determine the diversity and biological relevance of UV-resistance mechanisms in plants. A number of UV-sensitive mutants of both the single-celled alga *Chlamydomonas reinhardtii* and the model higher plant *Arabidopsis thaliana* have already been isolated. Although the underlying cause of the UV-sensitive phenotype of many of the *Arabidopsis* mutants remains to be determined, most of the UV-sensitive mutants are defective in DNA repair. Mutants defective in dark repair (*uvh1*, *uvr1*, *uvr5*, and *uvr7*) and in the photoreactivation of CPDs (*uvr2*) and (6-4)PPs (*uvr3*) have been identified and, with the exception of *uvr3*, mapped, and the genes corresponding to the photolyase mutations have been cloned and sequenced. The genes

corresponding to the dark repair mutations, presumably homologs of the excision repair genes of yeast and mammals, have not yet been isolated. The repair mutants themselves will provide a useful genetic background for the classical genetic analysis of DNA damage tolerance pathways (1–7).

Although the following protocol is designed for use with *Arabidopsis*, it can be applied to any plant species. However, *Arabidopsis* is the preferred model species for reasons that have been frequently described in the literature (8). In brief, *Arabidopsis* plants are small, prolific, have a short generation time, and are self-pollinating (though crosses are possible). *Arabidopsis* has a well-developed genetic map, with hundreds of molecular and physical markers. The *Arabidopsis* genome is unusually small for a higher plant (approx 100 Mbp) and is currently being sequenced via an international collaborative effort. The tiny *Arabidopsis* seedling is reasonably transparent to longer-wavelength UV; dimers are induced throughout the tissue, providing some advantages in terms of the measurement of DNA damage induction and repair.

2. Materials

2.1. EMS Mutagenesis/UV Sensitivity Screen

1. Ethyl methanesulfonate (EMS) (Sigma, St. Louis, MO, EMS is listed as methanesulfonic acid ethyl ester in the Sigma catalog). **Caution:** EMS is reactive and volatile. Use it in a fume hood, and wear gloves and a lab coat to protect yourself. EMS-contaminated material can be detoxified with 1 M NaOH. Bottles of EMS, once opened, should be protected from air by wrapping the tops in parafilm.
2. Deionized, sterilized H₂O.
3. Pots filled with Sunshine mix #2 (Sun Gro Horticulture, Canada) set in 1 × 2 ft sq. no-holes flats with clear, protective dome covers (Hummert International, Earth City, MO).
4. 1X *Arabidopsis* nutrients (9): 5 mL 1 M KNO₃, 2.5 mL 1 M KH₂PO₄ (pH 6.3), 2 mL 1 M MgSO₄, 2 mL 1 M Ca(NO₃)₂, 2.5 mL 20 mM Fe · EDTA, 1 mL of micronutrients, 985 mL of dH₂O.
5. *Arabidopsis* nutrient agar plates (1X *Arabidopsis* nutrients, no sucrose, 1% Bacto-agar).
6. Micronutrient stock solution: 70 mM H₃BO₃, 14 mM MnCl₂, 0.5 mM CuSO₄, 1 mM ZnSO₄, 0.2 mM NaMoO₄, 10 mM NaCl, 0.01 mM CoCl₂.
7. Germicidal (UV-C) lamp (Fisher Scientific, Pittsburgh, PA).
8. UV-C meter (Fisher Scientific, Pittsburgh, PA).
9. Orange polyvinyl chloride (PVC) film.

2.2. Pyrimidine Dimer Repair Assay

Growth and irradiation of seedlings:

1. Sterilization solution: Mix 2 mL of bleach (5.25% sodium hypochlorite), 50 µL of 20% Triton X-100, and 8 mL of dH₂O.

2. UV-B light source: UV transilluminator (e.g., model TM-20, UV Products, San Gabriel, CA).
3. UV-B-specific probe (UV Products).
4. Cellulose acetate sheet, 0.005 mil.
5. Aluminum foil.

DNA extraction:

1. CTAB buffer: 2% cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 0.2% (v/v) β -mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0.
2. Chloroform/isoamyl alcohol (24:1).
3. Isopropanol.
4. Tris/EDTA: 10 mM Tris-HCl, 1 mM EDTA, pH 7.5.
5. DNase-free RNase A (1 mg/mL).
6. 4.4 M ammonium acetate, pH 7.7.
7. Wash buffer: 76% ethanol, 10 mM ammonium acetate.

3. Methods

3.1. EMS Mutagenesis of *Arabidopsis* Seeds (see Note 1)

Mutagenesis is usually performed on the seeds; the resulting plants (termed the M1 generation) are of course chimeric and heterozygous, but these plants will spontaneously self-pollinate and produce some homozygotes in the M2 generation. For this reason, it is very easy to generate large M2 populations segregating for random mutations. These populations can then be screened for mutant phenotypes as described below. A very high density of mutations can be induced using the alkylating agent EMS; in a highly mutagenized, diverse population, one would, on average, expect to find a mutation in a particular target gene in one of every 3000 M2 plants. This high density of mutations (especially when compared with mutations induced by ionizing radiation, transposable elements, or T-DNA tagging) makes EMS the mutagenic agent of choice for very laborious screens, or for generating “proof of concept” mutations, i.e., demonstrating that a particular mutant class exists. If the screen or selection strategy is simple (i.e., there is an obvious visible phenotype, and no manipulations are required), and a goal is to clone the targeted genes, then we would recommend screening T-DNA tagged or transposable-element tagged populations. Some T-DNA tagged lines are available from the Ohio State *Arabidopsis* Biological Resource Center, and additional tagged lines are constantly being generated in various labs around the world.

1. Preparation of the flats. *Arabidopsis* seedlings require a constantly moist, but not waterlogged soil. Make sure the soil is moist before planting; subirrigate the soil the day before planting. We use Sunshine mix #2 as a growth medium, continuously subirrigated with distilled water. This substrate is easily wet provided it is never allowed to become completely dry. Do not compact this soil by pushing

- down on it or by irrigating from above. We generally use 2-in. deep, no-holes 2 × 1 ft sq. flats filled with 10 2-in. deep $5^{1/4} \times 4^{3/16}$ sq. in. pots. Allow the soil to take up moisture overnight; usually a flat will require about 3.5 L of dH₂O.
2. Add 120 μ L of EMS to 40 mL of ice-cold water in a 50-mL plastic disposable tube (final concentration 0.3% v/v). Dilute solutions of EMS in H₂O seem to be fairly stable over the period of a day; the mutagenic effects on *Arabidopsis* seeds have been found to be directly proportional to the product of concentration and exposure time (**10**).
 3. Weigh out 100 mg of dry seeds (about 7000 seeds). Do not chemically surface-sterilize the seeds; chemical treatment will have a marked and unpredictable effect on mutagenesis. Mix the seeds with 0.3% EMS solution. Cap the tube tightly and then shake well. Set the tube in a slow shaker in a fume hood for 16 h. Set up a small sample of seeds (approx 100) in dH₂O as a control for the effects of EMS on germination.
 4. Pour off the EMS solution into a waste beaker containing 1 M NaOH. Wash the seeds with sterile water (swirl and centrifuge briefly); repeat five times.
 5. EMS-treated seeds can be planted by suspending in water and pipeting onto the surface of the soil. Make a very dilute suspension of seeds, so that all of the seeds are well spaced in the pot. Approximately 1000 seeds can be planted in a single 1 × 2 ft sq. flat. Cover the flat with a clear protective dome until the true leaves have emerged (*see Note 2*). Incubate the flats at 4°C for 2 d, and then move into a continuously lit (approx PAR = 100 μ mol/m²s), 22°C growth room (*see Note 3*). Sow approx 100 of the mutagenized seeds onto a nutrient agar dish in order to determine the percent germination (the seeds are difficult to see in the soil); compare these with your dH₂O control (*see Note 4*).
 6. As the plants mature and set seed, they will spontaneously senesce and gradually dry out. They can be harvested any time after the siliques begin to shatter. M1 plants should be harvested in bulk, in single flat batches. Harvest the plants by cutting the shoots off at their base with a pair of scissors, and then leaving the entire plant tops to dry for two wk in a paper bag. Make sure any holes in the folds of the bag are taped shut, but leave the tops of the bags open so that air can circulate; wet plants will become moldy. After the 2-wk maturation/drying period, the M2 seeds can then be separated from the bulk of the M1 debris by sifting them through a fine wire mesh (e.g., a kitchen strainer). We store our seeds at room temperature in paper coin envelopes. Provided the seeds are not exposed to extreme variations in temperature and humidity, they should be good for several years.

3.2. Screening M2 Individuals for UV Sensitivity

Our original screen for UV sensitivity was a “root-bending” assay in which we screened many M2 families for growth with and without a challenge dose of UV (“M2 families” are the collective progeny [M3 seeds] of a single self-pollinated M2 plant) (**1**). The protocol presented below is a streamlined version of that originally developed by the Mount lab in which M2 individuals, rather than the progeny of M2 individuals, are screened (**3**). The screen is modi-

fied in that we have eliminated the use of foam as a UV-protective agent. The benefit of this screen is that it is very simple to perform; setup, UV treatment, and scanning for sensitive plants require relatively little time, effort, or attention to detail. The drawbacks, relative to the root-bending assay, are the frequent isolation of nonheritable phenocopies and the remote possibility that extremely UV-sensitive plants might be killed by the challenge dose. However, even given the large number of false-positives isolated, we highly recommend this M2 screen, rather than the labor-intensive root-bending screen.

1. Sprinkle dry M2 seeds onto the surface of the pots (prepared as described in **Subheading 3.1.**) at a density of about 100 seeds/pot (*see Note 5*). Cover with transparent domes and store at 4°C for two days.
2. Transfer to 22°C under cool white lamps filtered with Mylar or UV Plexiglas. Allow growth for about 2 wk. Remove the domes when the first true leaves (not the cotyledons) have emerged.
3. Irradiate the seedlings with a dose of 200 J/m² UV-C from an unfiltered germicidal lamp (*see Note 6*). To eliminate photoreactivation of UV-induced dimers, it is important to avoid exposure to blue or UV-A radiation after the UV treatment. Set up your germicidal lamp in a dark room with a red safety light. Allow the UV lamp to warm up for 15 min prior to use; its spectral qualities will change and then stabilize during this time (**II**). **Caution:** Protect your skin, especially your eyes and lips, from UV-C radiation by using a plastic face shield, lab coat, and gloves.
4. After exposing the pots (usually two at a time) to the challenge dose, transfer to a gold light environment (i.e., free of blue and UV-A wavelengths). Even transient exposure to blue light will drastically affect the reproducibility of the UV-induced effects. We use boxes with orange polyvinyl chloride lids and transport the irradiated seedlings from the dark room to the growth chamber in these boxes.
5. After 4–5 d of growth in the absence of photoreactivating light, transfer the plants to Mylar-filtered white light and check the plants over the course of a week for signs of UV sensitivity. These include browned, yellowed, puckered, or smaller than normal cotyledons and leaves. As the plant recovers from the UV treatment, the UV-sensitive phenotype will become easier to distinguish from a generically sickly mutant as the newly emerged (post-UV) leaves of a true UV-sensitive mutant will be healthy—very green and slightly moist looking. Apparently the pre-existing leaves are opaque to UV-C, and shield the developing tissues of the emerging leaves and the apical meristem from the radiation's damaging effects. Mark any putative mutants ("putants") by inserting a toothpick nearby in the soil.
6. Most UV-sensitive plants will recover from the challenge dose and set seed (more exquisitely sensitive plants might be isolated by using a lower challenge dose). If the pots are crowded, clear the wild-type plants away from the putative mutants by trimming the undesirable plants off at their base. This will make the identification of the putants easier as the plants grow larger and fill the pots. When the

putants begin to shed seeds, pinch them off at their base, and place the entire plant into a paper coin envelope. Allow the plants to dry down for at least 2 wk before attempting to germinate the seed. There is no need to clean the seed away from the rest of the plant; simply tip the coin envelope, and the seeds will roll out while the rest of the debris remains in the envelope.

7. Confirm the heritability of the UV-sensitive phenotype by checking the progeny of putative mutants for UV sensitivity. Prepare pots as described for the original screen and sow approx 50 seeds from a single putant family/4 × 5 sq. in. pot. Plant a pot of progenitor seeds as a negative control. Repeat the irradiation and recovery protocol described above, but in this case, UV-irradiate only 1/2 of each pot. UV-sensitive mutant families should display browning, puckering, and so forth, only on the irradiated side of the pot (*see Note 7*).

3.3. Assay of Pyrimidine Dimer Induction and Repair

Mutants should be characterized for UV transparency and the ability to repair UV-induced damage, e.g., via radioimmunoassay of dimers. The radioimmunoassay is described in Chapter 14; we describe below our procedure for the irradiation of seedlings and the extraction of DNA. The goal of the irradiation procedure is to produce as uniform a distribution of dimers in the plant material as possible. If dimers are induced only in the outermost layers of tissue and a large population of unirradiated cells remains, it would be difficult to distinguish between the elimination of dimers through DNA repair and the elimination of dimers via degradation of overly irradiated cells. The procedure below gives a random (Poisson) distribution of dimers (**12**). This is achieved by using longer-wavelength UV-B (which has better penetration characteristics than UV-C) and irradiating newly germinated *Arabidopsis* seedlings, which are very tiny (approx 0.1 mm in diameter) and fairly UV-B transparent. The resulting DNA is suitable for radioimmunoassay or the sequence-specific Bohr (Southern blot) assay for dimers (**13**). The DNA is cleavable with some, but not all, restriction enzymes. There is no need to separate newly replicated from older (preirradiation) DNA, since only a minor fraction of the cells in the seedling are actively dividing. We typically use 4 µg of DNA/(6-4)PP radioimmunoassay, and 1 µg of DNA per CPD radioimmunoassay, but the amount of DNA required will vary with the sensitivity of the antibodies. We use three 100 × 100 mm² Petri plates of seedlings, each sown with 15 mg of surface-sterilized seeds, for each time-point.

3.3.1. Irradiation of Seedlings

1. Weigh 10–15 mg of seeds into a 2-mL microcentrifuge tube. Add 1.5 mL of sterilization solution, and mix well by vortexing.
2. Incubate for 10 min at room temperature with occasional vortexing.
3. Pipet off the solution.

4. Wash with 1.5 mL of sterile dH₂O by resuspending the seeds, allowing them to settle, and pipeting off the wash solution. Repeat this wash three times.
5. Using a 1-mL pipetman, forcefully pipet the seeds onto the surface of a sterile *Arabidopsis* nutrient plate. It is critical that the seeds be uniformly distributed, or the seedlings will shade one another from UV. If the seeds form a clump, they can be dispersed by forcefully pipeting more sterile H₂O onto the plate. Once the seeds have settled on the surface of the plate in the desired pattern, remove the extra liquid using a pipet.
6. Store the plates at 4°C for 2 d, and then transfer to a 22°C growth room. Incubate the plates in a vertical position (e.g., with four plates taped together in a stack) so that the roots will grow across the surface of the agar. The agar is extremely UV-opaque, and it is important that the roots not grow into the agar.
7. Irradiate the seedlings approx 4 d after transfer to 22°C (see **Note 8**). Harvest the zero time-point immediately; wrap the repair time-point plates in aluminum foil (for dark repair assays), and return to the growth chamber.

3.3.2. Isolation of Seedling DNA

This procedure is designed for 0.5 g of seedlings, and should be scaled up or down accordingly. The protocol is a slightly modified version of the previously published CTAB procedure (14).

1. Preheat the CTAB extraction buffer to 60°C.
2. Harvest the seedlings in the absence of blue/UV-A light by scraping them off the plate onto a plastic weigh boat with a rubber policeman. Avoid digging into the agar. Weigh the seedlings, and place them in a chilled mortar and pestle containing liquid nitrogen (see **Note 9**).
3. Quickly grind the tissue to a fine powder. Scrape the powder into a prechilled 15-mL disposable plastic tube.
4. Add 5 mL of preheated CTAB buffer to the tube and mix well. Incubate the sample at 60°C for 30 min with occasional swirling.
5. Extract once with 5 mL of chloroform-isoamyl alcohol, mixing gently but thoroughly.
6. Spin at 1600g for 5 min at room temperature.
7. Transfer the aqueous phase to a clean 15-mL snap-cap tube. Add 2/3 vol of cold isopropanol, and mix gently for 1 h at room temperature to precipitate the DNA.
8. Spin in a table-top centrifuge (swinging bucket rotor) at the highest speed. Gently pour off as much of the supernatant as possible without losing the pellet, which will be very diffuse and loosely attached to the wall of the tube.
9. Resuspend the pellet in 500 µL TE buffer, and transfer to a 1.5-mL microcentrifuge tube. Add 5 µL of RNase A, and incubate for 30 min at 37°C.
10. Add 500 µL of chloroform/isoamyl alcohol and mix gently. Centrifuge at 13,000g for 2 min, and transfer the aqueous phase to a clean microcentrifuge tube.
11. Add 100 µL of 4.4 M ammonium acetate and 700 µL of cold isopropanol. Gently mix to precipitate the DNA.
12. Centrifuge at 13,000g for 5 min. Carefully remove the supernatant.

13. Add 1 mL of wash buffer directly to the pellet, and swirl gently to resuspend the DNA.
14. Centrifuge at 13,000g for 2 min, and carefully remove the supernatant. Vacuum dry the DNA until the scent of alcohol and ammonium acetate is gone, and then resuspend in 100 μ L of TE. Allow the sample to rehydrate for at least 24 h before quantifying the DNA concentration (see **Notes 10** and **11**).

4. Notes

1. EMS mutagenesis is easily over- or underdone: too little mutagenesis will mean that very large populations will have to be screened, and too much mutagenesis will result in lethality and sterility. Ideally, a very large M1 population (several thousand plants) should be harvested in bulk, and only a small fraction of the resulting M2 seeds screened for mutations, to avoid reisolation of particular alleles. Also, M1 plants should be harvested in “batches” from single pots or flats, so that different alleles of single genes can be positively identified as such (mutations in the same complementation group derived from a single pot are probably reisolates of a single mutation). The question of M1 vs M2 population sizes, and the frequency of M2 homozygotes derived from M1 chimeras has been debated in the literature (**15–18**). EMS mutagenesis involves a considerable amount of work, and there are often problems in achieving just the right level of mutagenesis. For this reason, we highly recommend that the investigator first look into purchasing mutagenized stock from Lehle Seeds (Box 2366, Round Rock, TX, or www.arabidopsis.com).
2. Because your desired mutants will have a UV-sensitive phenotype, it would be wise to shield the mutagenized population, even at the M1 stage, from the small UV component of most light sources by filtering the light through Mylar or UV Plexiglas (available from any plastics supplier; we use Golden State Plastics, Sacramento, CA). It is essential, of course, that your M2 plants be grown under filtered lamps.
3. At about 4 wk after germination, the plants can be counted and scored for sectoring. White, yellow, or pale green sectors, following the pattern of cell division, are a good indicator of mutagenesis. We have found that 5% sectoring indicates that the degree of mutagenesis was about right, but higher levels of sectoring indicate that the plants will be sterile. Unfortunately, the observation of “sectoring” can vary with the individual observer.
4. We have found that a dose that results in approx 80% seed germination (provided untreated seeds have 100% germination) usually indicates a nicely mutagenized population. However, some DNA repair mutants are unusually sensitive to either the toxic or mutagenic effects of EMS. If these mutants are used as a starting material, the optimal dose, and the relationship between mutagenesis and lethality must be derived empirically.
5. A good distribution of dry seeds can be achieved by folding and then unfolding an index card, placing the desired amount of seeds on the fold, and then tapping the card to shake the seeds onto the surface of the soil.
6. We use a dose of approx 1/3 the level required to see obvious stress in the parental line in the absence of photoreactivating light; for our stock (Landsberg *erecta*),

this challenge dose is about 200 J/m² UV-C from an unfiltered germicidal lamp. This value should be determined empirically for other stocks. The effects of UV-C are very distinctive and easy to spot: the distal ends of leaves are dead, but the proximal ends are healthy. Learn to identify this phenotype by treating the progenitor strain with increasing doses of UV and observing the results.

7. EMS-generated mutants will carry a large number of mutations. Lines displaying heritable UV-sensitive phenotypes should be immediately backcrossed for at least two generations (preferably more) to their progenitor line. The dominant or recessive nature (or possible cytoplasmic inheritance) of the mutation can also be determined at this point. The mutants can also be crossed to a line derived from a different ecotype for mapping of the mutation.
8. We irradiate in the darkroom under an inverted UV-transilluminator filtered through a fresh sheet of 0.005 inch thick cellulose acetate (to eliminate contaminating UV-C). The total UV-B dose, as measured with a UV-B-specific probe, is 1.5 kJ/m², at a dose rate of 30 W/m². This dose is sufficient to induce approx 60 CPD/Mb of single-stranded DNA. The concentration of dimers produced by UV-B radiation can vary widely with tissue thickness, degree of pigmentation (including chlorophyll concentration), and, just as importantly, light source. The numbers provided above were derived both from alkaline sucrose gradient and Bohr assay data.
9. Seedlings can be harvested off the plates, weighed, and frozen at -80°C for extraction at a later date.
10. Determination of DNA concentration is critical to the radioimmunoassay. In contrast, the degree to which the DNA is sheared has no effect on the accuracy of this assay. For this reason, length of DNA should be sacrificed for thoroughness of resuspension in preps destined for radioimmunoassay. Resuspend the rehydrated DNA via pipeting or swirling. Centrifuge out any insoluble material (13,000g for 5 min), transferring the solubilized DNA to a clean tube. We quantify our DNA via fluorometric assay, using a Hoefer fluorimeter (Hoefer, San Francisco, CA) following the manufacturer's instructions. This assay is insensitive to contaminating RNA and requires only nanograms of DNA per assay. Each assay is repeated three times, and the concentrations of all preps are double-checked by running an estimated 20 ng of DNA of each sample on an agarose gel, staining with ethidium bromide, and comparing the brightness of each lane.
11. DNA prepared for use with the Bohr assay for CPDs (**13**) should be treated carefully to avoid shearing, and the determination of exact concentration is not particularly critical. Seedlings grown for the Bohr assay should be sown on nutrient agar prepared with agarose, rather than Bacto-agar; DNA prepared from seedlings grown on Bacto-agar is difficult to digest with many restriction enzymes.

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Screening for γ -Ray Hypersensitive Mutants of *Arabidopsis*

Corinne S. Davies

1. Introduction

This chapter describes a method to isolate γ -ray hypersensitive mutants of *Arabidopsis thaliana* from ethyl methanesulfonate- (EMS) treated seed (**1**). The mutants are identified by visible symptoms of extreme radiation damage 10–15 d following exposure of seedlings to a threshold 10-krad dose (**Fig. 1**). Absence of meristem growth is the most visible symptom of extreme damage. Thus, it is used as the primary selection criterion. The method, therefore, targets single-gene EMS-induced mutations important for restoration of growth in radiation-damaged plants.

The key problem in any screen for radiation sensitivity is that the test dose is lethal or severely damaging. Thus, a method must be devised to rescue the mutants. In this screen (**Fig. 2**), this is accomplished by propagating nonirradiated siblings. The critical step is the generation of M1 seed pools. (M1 is the plant generation derived from EMS-treated seeds; a pool is obtained by collecting seeds from a self-pollinated M1 plant. The frequency of an EMS-induced homozygous recessive mutation within each pool is roughly 1:7, but it varies, depending on the number and subsequent cell divisions of the embryonic cells that produce the germ cells (**2**)). To test a pool, a random sample of 20–30 seedlings is exposed to γ -rays and carefully examined 10–15 d later for the presence of one or more hypersensitive seedlings (**Fig. 1C**). The irradiated seedlings are then discarded. To rescue the mutants, a second random sample of at least 30 nonirradiated sibling plants is planted and allowed to self-pollinate. The next generation is tested, again, by γ -ray exposure of seedling aliquots from single-plant pools. True-breeding M2 lines are then subjected to genetic analysis to confirm isolation of a single Mendelian gene. Using this

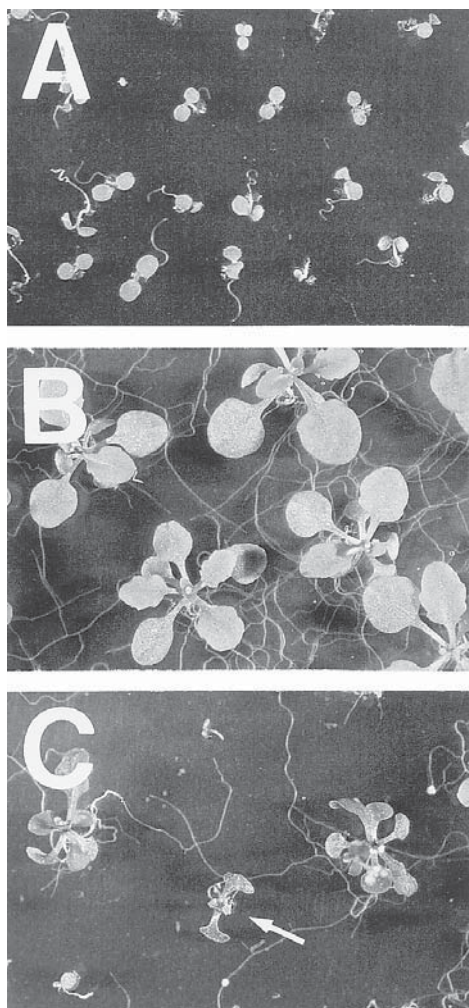


Fig. 1. Test system for evaluation of radiation damage in *Arabidopsis* (A). Appearance of 3- to 5-d-old seedlings at the time of γ -ray exposure (B). Growth of 15-d-old nonirradiated *Arabidopsis* plants for comparison (C). Growth inhibition of 15-d-old *rad* mutant (arrow) and wild-type plants by γ -rays (10 krad). Note difference in expansion of the cotyledons of the γ -ray hypersensitive mutant and "weak" plants that fail to grow for other reasons.

screen, we have isolated 12 mutants from a total of 3394 M1 pools. The mutants are fertile and thus can be maintained as true-breeding strains.

Advantages of this screen include simplicity of the irradiation procedure, and the ability to isolate and maintain any infertile mutants as heterozygotes. The latter was considered important, since yeast mutants hypersensitive to ion-

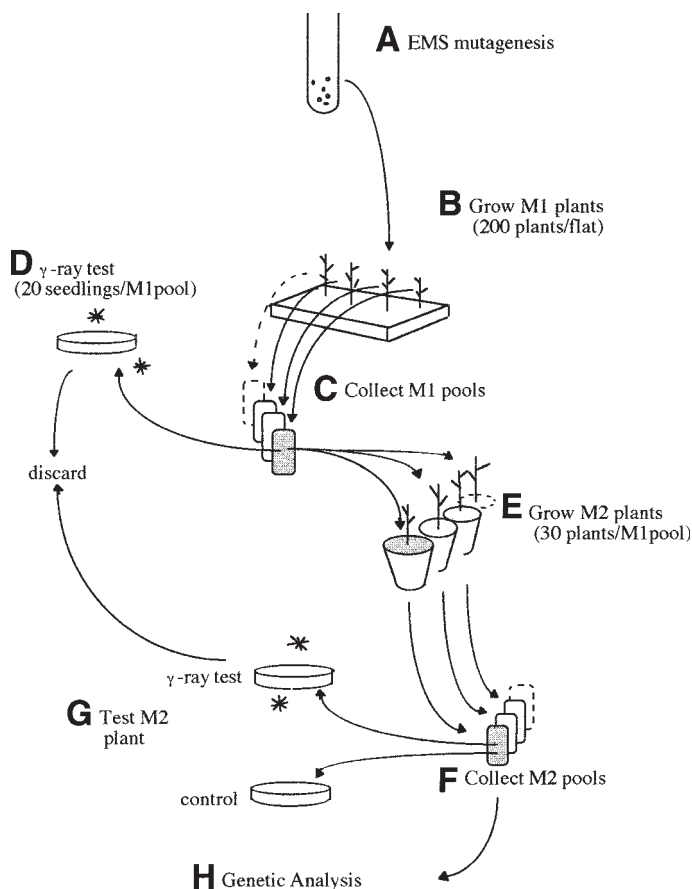


Fig. 2. Flow diagram of *Arabidopsis* mutant screen for γ -ray hypersensitivity.

izing radiation are frequently infertile (3). A disadvantage is the labor required to generate and label the seedling pools. This screening technique was devised for use with a ^{137}Cs experimental irradiator, which provides a uniform γ -ray field. The technique has also been used successfully with a point ^{60}Co source. An alternate screening strategy for use with X-rays was recently reported in which mutants are rescued by shielding their meristems (4).

2. Materials

1. γ -Ray source (see Note 1).
2. *Arabidopsis thaliana* seed: Seeds can be obtained from *Arabidopsis* stock centers or commercially (Lehle seeds) (see Note 2).
3. EMS mutagenesis: 0.5 N NaOH (to denature EMS), neoprene gloves, ethyl methanesulfonate (Sigma, St. Louis, MO; EMS is listed as methanesulfonic acid ethyl ester in Sigma's catalog).

4. Sterile culture: 100 × 15 mm sterile petri dishes, gas permeable tape, glucose, Murashige and Skoog (MS) salts (Sigma) or chemicals for fertilizer solution (*see Note 3*), 2 *N* KOH, 2 *N* HCl (for pH adjustment), Triton X-100 (20% solution), bleach (5.25% sodium hypochlorite).
5. Plant growth: potting soil (*see Note 4*), flats, pots, stakes, cool-white fluorescent lights or plant growth chamber.
6. Seed collection and storage: coin envelopes or Eppendorf tubes, desiccant (Drierite), cheesecloth.

3. Methods (*see Fig. 2*)

3.1. EMS Mutagenesis, Step A

Our *rad* mutants were isolated from EMS-treated M1 seeds obtained from a commercial source (Lehle Seeds). The following EMS seed treatment protocol has worked well for us in other mutant screens. **Caution:** EMS is a known carcinogen. Handle EMS in a fume hood only; wear gloves!

1. Weigh out 0.05 g of seeds.
2. Put the seeds into 15 mL of dH₂O, and mix.
3. Add 15–45 µL (0.1–0.3% v/v) EMS.
4. Mix and incubate overnight (12–15 h), rotating if possible.
5. Remove the EMS (put into 0.5 *N* NaOH, and dispose).
6. Rinse three times, and then rinse in 10 mL of dH₂O for 2–4 h.

3.2. Planting and Growth of EMS-Treated Seed, Step B

The purpose of this step is to grow to maturity a large population of EMS-treated plants in a manner that allows collection of seeds from individual plants. About 200 plants can be grown in a 10 × 20 in. sq. flat.

1. Add water to the soil mixture until the soil is moist, but not saturated. Add the moist soil mixture to a flat.
2. Dilute the EMS-treated seeds with excess water, swirl to suspend, and sow a few seeds at a time by drawing into a Pasteur pipet and applying dropwise to the soil surface. Try to space the seeds evenly.
3. Cover the flats with clear plastic wrap or a lid to retain moisture. Incubate at 4°C for 3 d to encourage uniform germination.
4. To grow the plants, place the flats in a growth chamber (*see Note 5*) or under cool-white fluorescent lights, and maintain at room temperature. Remove the cover when the plants germinate (after 3–4 d) to avoid etiolated seedlings. Keep the soil moist until the plants reach the four to five leaf stage.

3.3. Collection of M1 Pools, Step C

This step distinguishes our protocol from standard *Arabidopsis* mutant screens in which the progeny from M1 plants are pooled together. In our procedure, the seeds from each M1 plant form a pool.

1. Harvest the seeds promptly as soon as they mature by removing individual plants and shaking the stems that contain mature seeds over a sheet of paper. (The seeds are mature when the siliques turn yellow and their tops split open.) Separate the siliques from the seeds by filtering through a single layer of cheesecloth. Alternatively, the seeds can be harvested and threshed by a coin envelope technique (see **Note 6**).
2. Leave the seeds at room temperature for 1 wk to dry.

3.4. Testing M1 Pools for γ -Ray Hypersensitivity, Step D

The labor required for this step can be reduced by using nonsterilized seeds (see **Note 7**). It is also possible to use soil-grown plants for the irradiation treatment.

1. Prepare plates:
 - a. To 900 mL of dH₂O, add 1 package or 4.33 g of MS salts, and stir 10 min (see **Note 3**).
 - b. Adjust the pH to 5.8 using 2 N KOH and 2 N HCl.
 - c. Add H₂O to make 1 L, add 7.2 g of agar.
 - d. Autoclave, cool to 55°C, and pour into 100 × 15 mm Petri dishes.
 - e. Incubate the Petri dishes (with lids on) in a laminar hood overnight to dry.
2. Plant at least 10 M1 plant families in each Petri dish (20–30 plants/pool) by sprinkling the seeds from the coin envelope onto the agar surface. Use a high planting density to maximize the number of seedlings for each γ -ray exposure, but allow enough space between the seedlings so visual scoring of individual plants is possible.
3. Seal each dish with filter tape (see **Note 8**).
4. Incubate the dishes at 4°C for 3 d.
5. Incubate the dishes at room temperature under lights (or in a growth chamber).
6. Expose to γ -rays 3–5 d later (see **Note 9**). The seedlings should be uniformly germinated at the time of exposure (**Fig. 1A**).
7. Incubate as in **step 5**.
8. Examine each M1 plant pool carefully 10–15 d after irradiation. Select all pools that exhibit at least one hypersensitive seedling (**Fig. 1C**).

3.5. Grow M2 Plants, Step E

The M2 plants are grown in separate pots to eliminate cross-contamination of seeds.

1. Plant at least 30 seeds (1 seed/pot) from each selected M1 plant family, and grow in a growth chamber or at room temperature under fluorescent lights. Grow the plants in an area free of stray *Arabidopsis* seeds. Water the plants by subirrigation. Add fertilizer as needed.
2. Separate the pots when the plants have started to bolt (see **Note 10**).

3.6. Collect M2 Pools, Step F

Harvest and dry the seeds from each M2 plant as described in **Subheading 3.4**.

3.7. γ -Ray Test the M2 Pools, Step G

The purposes of this step are to evaluate carefully the M2 pools for the presence of hypersensitive plants and to eliminate false-positives. The seedlings are grown on agar under sterile conditions and are spaced for maximum visibility (**Fig. 1A**).

1. Prepare plates as described in **Subheading 3.4**.
2. Sterilize the seeds (*see Note 11*):
 - a. Mix 40 mL of bleach with 40 mL of sterile dH₂O, and add 80 μ L of a 20% Triton X-100 stock solution.
 - b. Place seeds into a sterile container, add bleach solution.
 - c. Incubate for 3–5 min, and swirl occasionally to moisten all the seeds.
 - d. Remove the bleach solution and rinse four times with sterile water.
3. Draw the sterilized seeds and water into a sterile Pasteur pipet (with a cotton plug at the bulb end to prevent contamination), and plant one-by-one onto the agar surface (*see Note 12*).
4. Plant duplicate sets of each M2 pool. Treat both sets as described in steps 3–6 of **Subheading 3.4**, except do not expose one of the sets to γ -rays.
5. Examine each plate carefully 10–15 d after irradiation, record the number of wild-type and hypersensitive plants within each pool, and test for conformity to Mendelian expectations. Discard any pool exhibiting symptoms of radiation damage in the absence of radiation (false-positives). Select the true-breeding wild-type and hypersensitive pools for further analysis. If no true-breeding pools are found, select heterozygotes if present.

3.8. Genetic Analysis to Identify γ -Ray-Sensitive Mutants of Arabidopsis, Step H

Classical genetic analysis is used to confirm isolation of a single Mendelian gene and to map the gene to its chromosomal location. The following is a brief summary of the crosses we performed for these purposes. The reader is referred elsewhere for details about these standard procedures (**5**).

1. Plant seeds from the M2 pools (obtained in step G), stocks of the parental strain, and a contrasting ecotype. Grow plants to the flowering stage.
2. Do reciprocal crosses between mutant plants and the parental strain to test for conformity to Mendelian ratios, to generate backcrossed stocks, and to search for cytoplasmic effects.
3. Do crosses between the mutant lines and the contrasting ecotype to generate populations for mapping and to study genetic penetrance of the mutant gene.
4. Collect the selfed-seed from mutant and wild-type plants to generate stocks and to confirm genetic transmission of the *rad* mutant.

4. Notes

1. We used the following irradiators: McGill University Medical School ^{137}Cs source, 108–122 rad/min (Gamma Cell 40 Irradiator, Nordion, Ottawa, Canada), and University of Arizona Cancer Center ^{60}Co source, 90 rad/min (Theratron 80, Atomic Energy of Canada).
2. *Arabidopsis* seeds can be obtained from the following sources:
 - a. *Arabidopsis* Biological Resource Center
Web site: <http://aims.cps.msu.edu/aims/>
e-mail: arabidopsis+@osu.edu
phone: 614-292-9371
Fax: 614-292-0603
 - b. The Nottingham *Arabidopsis* Stock Centre
Web site: <http://nasc.nott.ac.uk/>
e-mail: arabidopsis@nottingham.ac.uk
 - c. Lehle seeds
Web site: <http://www.arabidopsis.com/>
e-mail: webmaster@arabidopsis.com
Phone: 512-388-3945,
Fax: 512-388-3974
PO Box 2366
Round Rock, TX
78680-2366 USA
3. An alternative fertilizer: Add 5 mL from each of the following stock solutions to 1 L of dH_2O : $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (73.96 g/L), $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (94.44 g/L), $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (48.51 g/L), KNO_3 (60.66 g/L), and Na_2EDTA (5.0 g/L) + $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.48 g/L). Add trace minerals if prepared with ultrapure water. Adjust to pH 5.8 if used to prepare nutrient agar. This fertilizer also works well with soil-grown plants.
4. It is possible to grow *Arabidopsis* on most commercial potting soils, but it prefers a well-aerated soil and a dilute fertilizer solution.
5. We obtained best plant growth in a growth chamber at 70% relative humidity, using autoclaved soil, filter-sterilized fertilizer solution (*see Note 3*), a 16-h photoperiod, and a combination of incandescent and fluorescent light bulbs (6000 lx). Under these conditions, it is possible to attain a 6-wk generation time and high uniformity of plant growth. *Arabidopsis* plants can also be grown successfully almost anywhere indoors at room temperature and under cool-white fluorescent bulbs, but these conditions can produce variable results.
6. Coin envelopes have the advantage that they allow the seeds to respire. They also provide a space to record information about the seeds, and they can be sorted and stored easily. The envelopes must be taped on the bottom to prevent seed leakage. A shortcut method for seed harvest is to place the plant shoot into the envelope, let it dry for a week, crush the package with your fingers to thresh the seeds, and collect the seeds by making a crease in the envelope flap and tilting the envelope downward toward the flap while tapping lightly. The seeds will flow to form a pool on the envelope flap, and the other plant parts will remain in the envelope.

The seeds can also be stored in Eppendorf tubes that have a small hole punched on their lid. We have obtained good germination rates with seeds stored for over 6 yr at 4°C and under desiccation.

7. Fungal growth can be slowed by eliminating all carbon nutrient sources from the agar medium and by using seeds that have been promptly harvested, threshed, and then stored under desiccation. Fungal growth is also minimized by the γ -ray treatment itself.
8. Do not use nonporous tape or Parafilm to seal the plates. Plant growth is inhibited when the air supply is cut off.
9. The higher-energy photons from the ^{60}Co source require a bolus or buildup material to achieve dosage (6). In Petri dish-grown plants, the agar provides a sufficient buildup material, and thus the plates are inverted relative to the source during the irradiation procedure. When soil-grown plants are irradiated, a 1/4-in. bolus is layered over the top of the pots.
10. Commercially available *Arabidopsis* growth systems “Arasystem” and “Arabi-Patch” can be used to separate the bolted plants (Lehle seeds; see **Note 2**). It is also possible to devise your own system from various household and lab items.
11. About 20 small batches of seeds can be sterilized simultaneously by using a small volume of the bleach solution relative to the size of the container, incubating for the required 3–5 min, and diluting all of the batches quickly to a nonlethal concentration with an excess of sterile water.
12. A single layer of sterile cheesecloth can be placed on the surface of the agar to separate the seeds during planting. Also, plant growth under sterile conditions can be enhanced by addition of 5 g of glucose/L of medium.

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Isolation of Mutagen-Sensitive Chinese Hamster Cell Lines by Replica Plating

Malgorzata Z. Zdzienicka

1. Introduction

Cell lines with an increased sensitivity to mutagens, such as ultraviolet (UV) light, X-rays, alkylating compounds, and crosslinking agents, are defective in a cellular response to these agents. These responses include mechanisms that process DNA lesions, scavenge free radicals, or regulate cell-cycle progression. The molecular defects in cell lines derived from patients with inherited recessive disorders that combine cancer proneness with an abnormal response to DNA-damaging agents, such as xeroderma pigmentosum, ataxia telangiectasia, and Fanconi anemia, have been extensively studied (*see*, for example, Chapters 7, 9, 29, and 44). However, such human diseases may not identify all possible cellular responses to mutagenic treatments, since only those defects that are manifested at the clinical level, and not lethal *in vivo*, can be detected. Therefore, in addition, many mutagen-sensitive mutants have been obtained in rodent cell lines. The availability of such mutants is essential to identify the genes involved, their products and functions, as well as to assess the biological consequences of their impact. It has also become evident that rodent cell mutants defective in DNA repair provide an important tool for the isolation of human genes complementing the defect in these mutants (*see* Chapter 7). Therefore, to dissect the cellular response to a specific mutagen, it is essential to have a comprehensive set of mutants with an increased sensitivity to the agent.

To induce such mutants, “wild-type” cells are treated with a strong mutagen, such as ethyl nitrosourea (ENU) or ethyl methanesulfonate (EMS), following which the mutagenized cell population is screened to identify clones with an increased sensitivity to the desired DNA-damaging agent(s). To test a large

number of clones, the replica-plating technique has been used successfully in our laboratory (**1**), and many mutants with increased sensitivity to these agents have been isolated in hamster V79 or Chinese hamster ovary (CHO) cell lines (**1–7**). The replica-plating technique is a standard method used in microbial genetics where many clones are screened. Cells from the mutagenized population are plated in plastic tissue-culture dishes. When the single cell-derived clones are visible to the eye, they are transferred to 96-well master plates and cultured for several days. Cells from the master plate are then used to make replicas of the 96 lines, whereupon each replica is treated with a mutagen at a dose that is only marginally toxic to wild-type cells. Sensitive clones are identified by comparing the control replica with the mutagen-treated replica, since sensitive clones show growth retardation. The main advantage of this technique is that it enables cells derived from a single colony to be screened for hypersensitivity to a number of different mutagenic agents. Once such mutants are identified, they should be examined for their sensitivity to other mutagenic agents, and characterized to determine whether they represent new complementation groups.

2. Materials

1. Cells: CHO or V79 cells (*see Note 1*).
2. Ham's F10 medium: modified by omission of hypoxanthine and supplemented with 10% fetal calf serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin. This is referred to as "standard" medium (*see Note 2*).
3. Standard medium supplemented with 20 mM HEPES, pH 7.4.
4. Phosphate-buffered saline (PBS), pH 7.4.
5. Trypsin solution: 0.25% trypsin, 0.02% EDTA in PBS.
6. Cryo tubes, 1.8 mL (Nunc).
7. Tissue-culture dishes: 10- and 15-cm (e.g., Greiner).
8. 96-Well microtiter plates with flat bottoms (Costar).
9. Multichannel pipeter for dispensing liquids (20–200 μ L) into 96-well plates.
10. Transtar-96[®] portable liquid handling system; 96-tip sterile, disposable cartridges (Costar). (*See Note 3*.)
11. 0.9% NaCl solution.
12. 0.2% Methylene blue (Sigma, St. Louis, MO) solution: Dissolve 2 g of methylene blue in a few microliters of ethanol, and then add water to 1 L.
13. 0.4 M ENU (PFaltz & Bauer, Waterbury) freshly prepared in dimethyl sulfoxide (DMSO).
14. EMS (Kodak, Tramedico bv. Weesp, Holland), freshly dissolved in PBS (1% v/v; 94 mM). Vortex to dissolve.
15. Methyl methanesulfonate (MMS) (Merck): Prepare as for EMS. (1% v/v; 118 mM).
16. Mitomycin C (MMC) (Kyowa Hakko, Kogyo Co. Ltd. Tokyo, Japan), stock solution (4 mg/mL): Dissolve in sterile H₂O (or PBS). Keep at 0–4°C.
17. Bleomycin (BLM) (Lundbeck B. V. Amsterdam, Holland), freshly dissolved in PBS.

3. Methods

Culture cells in 10- or 15-cm plastic dishes containing 10 or 25 mL of standard medium, respectively, at 37°C in an atmosphere of 5% CO₂ in a humidified incubator.

3.1. ENU Mutagenesis

1. Treat a suspension of $>10^7$ cells with freshly prepared ENU (4 mM final concentration) in 10 mL of prewarmed medium supplemented with 20 mM HEPES. Incubate at 37°C for 1 h. This should give a surviving fraction of approx 1% (see **step 3**).
2. Collect the cells by low-speed centrifugation (135g), wash twice with PBS, and resuspend in 10 mL of standard medium.
3. To assess the level of killing by ENU, seed 100–1000 treated cells in a 10-cm dish. At the same time, seed ~100 cells from the untreated population to serve as a control. Incubate both dishes for 8–10 d. Rinse with 0.9% NaCl solution, air-dry, and stain with methylene blue solution. Count the visible colonies.
4. Seed the remaining treated cells in two or three 15-cm dishes. After 4 d of incubation, trypsinize the cells, and collect by low-speed centrifugation. Set aside $\sim 5 \times 10^5$ cells to assess the degree of mutagenesis as described in **Subheading 3.2**. To the rest of the cells add DMSO to a final concentration of 6%. Aliquot 10^6 cells/Cryo tube, and transfer the tubes to a –100°C freezer. After 1 d, the tubes can be transferred to liquid nitrogen for long-term storage. Each plate will yield about 7–10 tubes.

3.2. Estimation of the Level of Mutagenesis

To evaluate the degree of mutagenesis, the frequency of induced *hprt* mutants in the mutagenized population is determined by incubating the cells in standard medium containing 6-thioguanine (TG) (see **Note 4**).

1. Incubate 5×10^5 cells from **step 4** of **Subheading 3.1**. for an additional 4 d (8 d in total).
2. Trypsinize the cells, and collect by low-speed centrifugation.
3. Seed 10^5 cells in each of three to five 10-cm dishes containing medium supplemented with 7 µg/mL of TG. Seed 200 cells in each of three to five dishes containing standard medium (without TG) to determine the number of viable cells.
4. After 10 d of incubation, rinse the dishes with 0.9% NaCl, air-dry, and stain with methylene blue. Count the visible colonies and calculate the frequency of TG-resistant (*hprt*^r) mutants. There should be at least 1 *hprt*^r mutant/ 10^3 viable cells.

3.3. Screening for Mutants by Replica Plating

1. For each mutant isolation, dilute an ampule of frozen cells (from **step 4** of **Subheading 3.1**.) in standard medium and seed to obtain about 20–50 single cell-derived clones/10-cm dish.

2. After 8–12 d of incubation, remove the medium, pick up cells from each colony with the flat end of a sterile wooden toothpick and transfer to a 96-well microtiter master plate whose wells each contain 25 μL of trypsin solution. Trypsinize the cells for 3 min at 37°C, and then add 200 μL of standard medium (*see Note 5*). Return the plates to the incubator.
3. After about one week, remove the medium, wash the cells with PBS, add trypsin solution for 3 min at 37°C (*see Note 5*), and add 200 μL of standard medium. Mix the cells by shaking the master plate.
4. Using a Transtar-96 pipeter, transfer cells from the master plate to three to six replica plates containing standard medium (*see Note 6*). One plate will serve as a control for retrieving mutants, a second control plate will be stained with methylene blue, and the remaining replica plates will be treated with mutagens.
5. Irradiate or treat with a chemical mutagen at the following doses, which allow 80–100% survival of wild-type cells:
 - a. 254 nm UV light (6 J/m²): 4 h after transfer, when the cells are attached, remove the medium, wash the cells with PBS, irradiate, and then add back standard medium.
 - b. X-rays (2–3 Gy): Irradiate the cells in medium without waiting for attachment. There is no need to change the medium.
 - c. MMC (5 ng/mL): At **step 4**, transfer the cells to standard medium already containing MMC. Cells receive a continuous treatment.
 - d. EMS (2 mM): Treat as for MMC.
 - e. MMS (0.3 mM): Treat as for MMC.
6. After more than 1 wk of growth, rinse all except one of the control plates with NaCl solution, air-dry, and stain the cells with methylene blue.
7. Compare the stained control plate with the plates treated with mutagens. Those wells containing no cells, or greatly reduced numbers of cells, in the treated plates compared to the control plate are considered putative mutants.
8. Pick the putative mutants from the untreated control plate, grow them, and retest for sensitivity.
9. Reclone the sensitive mutant cells by seeding ~20 cells/10-cm dish (*see Note 7*). Isolate three subclones after 10 d, and test the sensitivity of these subclones.

3.4. Survival Experiment to Assess the Degree of Mutagen Sensitivity

1. Trypsinize the mutant cells in exponential growth, and plate ~300 cells into 10-cm dishes in triplicate.
2. After 4 h, when the cells are attached, treat with a mutagen over a range of doses. Use as a guide the doses given in **step 5** of **Subheading 3.3**. For UV irradiation, remove the medium, wash the cells with PBS, irradiate, and add back standard medium. For X-rays, irradiate the cells in standard medium without changing it. After chemical treatment, wash the cells twice with PBS, and add fresh medium. Incubate the cells for 8–10 d to obtain colonies visible by eye.

3. Rinse the dishes with NaCl solution, air-dry, stain with methylene blue, and count visible colonies.

3.5. Characterization of Mutants

1. To determine the stability of the isolated mutant, culture the mutant cells for 2–3 mo. After 1, 2, and 3 mo, re-examine the sensitivity of the mutant over a range of doses. A tailing curve may indicate the presence of revertants in the cell culture. In this event, reclone and retest the mutant.
2. To assess the degree of sensitivity of the mutant to other mutagenic agents, perform cell-survival studies after treatment with mutagens from at least four different classes of DNA-damaging agent:
 - a. UV.
 - b. X-rays, BLM.
 - c. Monofunctional alkylating agents, such as MMS, EMS, and ENU.
 - d. Bifunctional alkylating (cross-linking) agents, such as MMC and cis-dichlorodiammine platinum(II) (cisplatin; cis-DDP). Compare the D_{10} values (i.e., the dose required to kill 90% of the cells) of the parental and mutant cells (*see Note 8*).
3. To determine whether the isolated mutant represents a new complementation group, perform a genetic complementation analysis with representative mutants of different extant complementation groups as described in ref. (3,4).

4. Notes

1. Cell lines used for the isolation of mutants must be pseudo-diploid. They cannot be strictly diploid, since the probability of inducing mutations in two alleles is extremely low. Therefore, mutants can only be induced in cells that are functionally or structurally hemizygous. Different Chinese hamster cell lines, even of the same origin, but growing for several years in different laboratories, may show different hemizygosity, thus allowing mutants defective in different genes to be isolated. To obtain new complementation groups of mutants, use cell lines which have not been used extensively for the isolation of such mutants. We have found that mutagen-sensitive cells were obtained with a 10-fold higher frequency from V79 than from CHO9 cells. Most probably this is owing to the different extent of hemizygosity in these two “wild-type” cell lines. V79 cell lines cultured for a long time in different laboratories have different hemizygosity.
2. Other types of culture medium may be used. However, the cells should first be adapted to the medium. Medium lacking hypoxanthine is required to select for 6-thioguanine-resistant mutants (*see Subheading 3.2.*).
3. When a Transtar-96 multipipeter is not available, use the sterile cartridges as “stamps” to transfer cells.
4. *hprt* is an X-linked gene encoding a nonessential purine salvage pathway enzyme, hypoxanthine phosphoribosyl transferase (HPRT). HPRT metabolizes TG to a cytotoxic nucleotide. *See ref. (8)* for a detailed discussion of *hprt* mutagenesis.

5. When preparing the master plate, transfer no more than 12–24 clones to a part of the 96-well plate, trypsinize them, and add standard medium before transferring the next 12–24 clones. Cells will be killed if kept in trypsin for too long. Do the transfers quickly and do not have too many clones in trypsin at any one time.
6. An alternative method of transferring cells from the master plate to the replica plate employs Cytodex-1 microcarrier beads (Pharmacia) (9). However, this method cannot be used to screen for UV-sensitive clones because of shielding from the beads. When cells in the replica plate form colonies, add medium containing 600 beads, which gives a monolayer of beads covering the bottom of each well. Incubate for two additional days to allow the cells to grow onto the microcarrier beads. Make replicas of each plate by transferring medium containing the suspended beads with attached cells.
7. All mutagen-sensitive lines should be recloned to avoid possible contamination with other cells.
8. For further studies, use cells with a significantly increased sensitivity to the given agent. To date, the isolated mutants show a 2- to 10-fold increased sensitivity toward X-rays or UV radiation, and more than 10-fold to MMC.

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Strategies for Cloning Mammalian DNA Repair Genes

Larry H. Thompson

1. Introduction

During the last 20 years, the cloning and identification of DNA repair genes in bacteria, yeast, human, mouse, and other organisms have been an expanding enterprise. Gene/cDNA isolation and analysis is a critical step toward discovering gene/protein function by enabling subsequent characterization of over-expressed and purified recombinant protein, and by providing information about gene structure that can be used to prepare gene targeting vectors. Although most of the human genes have been identified for nucleotide excision repair (NER) (*1*), there probably remain to be discovered numerous genes that act in recombinational repair and in processes that coordinate the cellular response to DNA damage with respect to the cell division cycle. Only a few human cell-cycle checkpoint genes have been reported, and mammalian genes that correspond closely in function to the *RAD6* epistasis group in *Saccharomyces cerevisiae* are notably lacking. Although two human homologs of *RAD6* were identified (*2*), their functions appear to involve chromatin modification rather than response to DNA damage (*3*). This chapter reviews the wide variety of gene cloning strategies, which have seen varying degrees of success, and comments on their relative merits.

A summary of the cloned human DNA repair genes is given in **Table 1**, which is arranged according to the type of pathway in which each gene acts. Some genes function in multiple repair processes as indicated. **Table 1** is intended to supplement the discussion without being repeatedly referred to.

2. Cloning of Genomic Sequences by Functional Complementation

Some of the first human DNA repair genes, primarily those in the NER pathway, were cloned on the basis of functional complementation by using rodent

cell mutants that have a specific DNA repair defect. In the late 1970s and early 1980s, as recently reviewed (4), the first Chinese hamster and mouse cell mutants displaying hypersensitivity to UV radiation, ionizing radiation, or chemical mutagens were reported. Chinese hamster ovary (CHO) cells proved to be especially suitable for gene cloning, because they efficiently incorporate exogenous DNA without extensive breakage of the transfected sequences (5). Integration of a human DNA repair gene into chromosomal DNA of mutant cells can confer resistance to a DNA-damaging agent damage (e.g., UV or mitomycin C [MMC]) to which the mutant cells are hypersensitive. In order to exclude revertants and enrich for genuine transformants, it was necessary to incorporate an additional selection condition. The commonly used selectable marker genes were the bacterial *gpt* gene (6), which confers resistance to mycophenolic acid, and microbial genes that behave as dominant markers in all cell types. The *neo* gene (7) provides resistance to Geneticin (also referred to as G418), and the *hyg* gene confers resistance to hygromycin B (8). Before transfection, human genomic DNA is mixed with, or ligated to, the DNA of a plasmid that carries one of these dominant genes that is expressed by a mammalian-virus promoter, as from SV40 virus. Because cells efficiently ligate the ends of transfected DNA prior to chromosomal integration (9), it is unnecessary to perform a specific ligation step in vitro before transfection.

The general strategy has been to introduce, into repair-deficient cells, genomic and dominant marker DNAs in the form of precipitates of calcium phosphate (10). Thus, cotransfection of genomic DNA and the marker gene will produce rare transformant colonies in which the selected repair gene and drug resistance are both expressed, as illustrated in **Fig. 1**. Transfer frequencies for dominant markers are in the range of 10^{-3} – 10^{-4} , and the frequency of cells expressing both drug resistance and the gene of interest is $\sim 10^{-7}$ in CHO cells.

Once a human repair gene is transferred into a hamster cell, a method of rescuing it must be developed. Many human genes carry copies of the abundant *Alu* family of repetitive sequences, which can provide a tag for tracking the repair gene. Since this repeat element is absent in the hamster genome, it serves as a specific marker for the human DNA fragment (**Fig. 1**). A primary transformant will likely contain extraneous human DNA sequences as well as dominant marker genes that are not closely linked to the repair gene. Therefore, it is necessary to perform a second round of transfection in which DNA from a primary transformant is introduced into mutant cells. Secondary transformants have a reduced probability of carrying either a dominant marker or *Alu* repeats at sites that are distant from the repair gene. Tight linkage of the repair gene to a dominant marker is optimized by reducing the

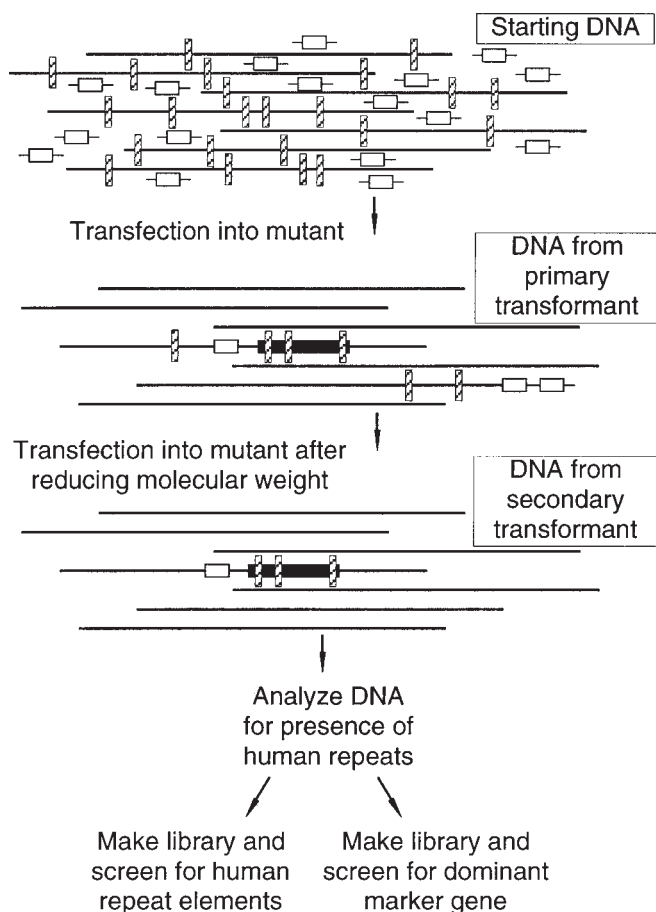


Fig. 1. Experimental strategy for isolating human repair genes by complementation of mutant cells with human genomic DNA. Human-specific repeat elements, such as *Alu*-family repeats, are illustrated by the small vertical shaded boxes in the genomic DNA (horizontal lines). A plasmid carrying a dominant marker (e.g., *neo*, *gpt*, *hyg*) is shown by the open rectangle, and the repair gene to be isolated is illustrated by the black rectangle.

molecular weight of the DNA by restriction enzyme digestion (11) or by mechanical shearing (12) before the primary and secondary transfections. Ideally, a secondary transformant will express both the dominant marker and repair gene, and will have the DNA configuration shown in **Fig. 1**, which is ready for cloning. At this stage, the DNA is analyzed by Southern blotting to check for the presence of *Alu*-family repeats and/or the dominant marker.

The *Alu* sequences can be detected using a cloned *Alu* family sequence (such as pBLUR8) (13) or reannealed human DNA of a Cot value of 1 as the probe.

In the earlier studies, gene rescue and cloning from transformants was done using cosmid vectors, which accept inserts in the 35–45 kb range. Large-insert bacterial vectors, namely P1 bacteriophage-derived artificial chromosome (PAC), and bacterial artificial chromosome (BAC), are now in routine use (14–16), and these can accept inserts of 100 kb and larger, which will accommodate most genes. *Escherichia coli* libraries are prepared by partial digestion of the DNA from a secondary transformant to the desired average insert size by using an enzyme, such as *Mbo*I, which has a 4-bp recognition site. After plating the transformed bacteria on filter paper for high-density colony formation, two replica filters are prepared from each master filter. The replicas are hybridized using ³²P-labeled DNA probes specific for the linked marker gene or *Alu* family repeats (17). Alignment of the two autoradiographic images from pairs of replica filters allows elimination of background spots, which can resemble true positives. A 15-cm diameter filter accommodates up to 10⁵ colonies each, which is equivalent to about one haploid genome when using cosmids. To ensure statistical coverage of a library, it is desirable to screen 5–10 diploid genome equivalents (10–20 pairs of filters). Positive clones are removed from master filters and purified by further growth, dilution, and successive screening (usually two additional rounds).

Genes that were isolated by functional complementation were *XPA* (18), *ERCC1* (11), *ERCC2/XPD* (12), *ERCC3/XPB* (19), *ERCC4* (20), *ERCC5/XPG* (21), *ERCC6/CSB* (22), and *XRCC1* (23). In the case of *ERCC4*, transfection was done using a chromosome-specific cosmid library of chromosome 16 to which the gene had been assigned. The chromosome 16 library was constructed in the vector sCos-1, which carries a *neo* gene that expresses in both mammalian and bacterial cells. Cloning of *ERCC4* from a secondary transformant was done by preparing a library in a second cosmid vector not having a *neo* gene. *ERCC4*-containing cosmids were selected by kanamycin resistance (conferred by the *neo* gene in sCos-1, which was directly linked to *ERCC4*). The *ERCC3*, *ERCC5*, and *ERCC6* genes proved to be too large (32–85 kb) or difficult to recover intact in single cosmid clones. In these cases, portions of the genes were recovered from cosmids and used as probes to obtain cDNAs.

A variety of mutagen-sensitive mutants have been isolated in mouse lymphoma cells, but these cells have very low DNA transfection efficiency (24). In one instance, it was possible to derive a transfection-proficient UV-sensitive line through a cell-fusion procedure (24), and the resulting line was used for cloning of *ERCC5* (25).

3. Cloning of cDNA Sequences by Functional Complementation with Expression Libraries

3.1. Transfection of cDNA Libraries into Human Cells

Earlier attempts to use established human cell lines for gene transfer were unsuccessful because human cells often do not stably incorporate foreign DNA (26). However, an alternative approach involving the use of episomally replicating shuttle vectors that contain the Epstein-Barr virus (EBV) origin of replication has circumvented this limitation. Human cells that stably maintain the *EBNA1* gene are efficient recipient cells (27) for transfection of a cDNA library in an EBV expression plasmid, such as the pEBS7 library of Peterson and Legerski (28). In the pEBS7 vector, the insert cDNAs are expressed using a cytomegalovirus (CMV) promoter. The vector used to prepare this library contains both an EBV *oriP* for episomal replication and also an *ori* for replication in *E. coli*. The ampicillin resistance gene allows for selection in *E. coli*, and the *hyg* (hygromycin B) gene is present for selection of transformants in mammalian cells. The *XPC* (29) and *CSB* (30) cDNAs were obtained using pEBS7 libraries. Although the initial *XPC* cDNA conferred full correction to UV resistance in XP group C cells, it was later found to be truncated at the 5'-end (31). A similar vector system was used to isolate the *FAA* and *FAC* cDNAs that correct lymphoblasts in Fanconi anemia complementation group A (32) and group C (33), respectively.

3.2 Transfection of cDNA Libraries into Hamster Cells

The large pEBS7 libraries (2×10^7 clones) developed by Legerski (28) were used by Thompson and coworkers in several studies with CHO cell mutants. The EBV shuttle vectors are generally thought not to replicate in rodent cells (27,34). Therefore, selection for resistance was based on the assumption of chromosomal integration of the correcting cDNA. A common feature of the three mutants used was their high sensitivity to killing (10- to 100-fold compared with wild-type cells) by MMC and their broad-spectrum sensitivity to other DNA-damaging agents, including ionizing radiation and UV radiation. Selection for primary transformants was performed by introducing supercoiled plasmid DNA in the form of calcium phosphate precipitates (10), and selecting simultaneously for resistance to MMC and hygromycin B. For two of the mutants, selection of secondary transformants was done by transfecting genomic DNA from primary transformants. Calcium phosphate precipitates were used instead of electroporation, because they are thought to favor the integration of larger amounts of DNA.

In each of the three studies, a different strategy was used to recover the correcting cDNA. For the mutant *irs1SF* (35), MMC selection was imposed on

the equivalent of $\sim 5 \times 10^5$ primary transformants, and two MMC-resistant clones were obtained (36). Four secondary transformants of *irs1SF* cells were recovered from 2.7×10^8 transfected cells. The correcting cDNA, designated *XRCC3*, was recovered from the DNA of one secondary transformant by screening a cosmid library for the linked *hyg* gene (36).

Transfection of the hamster V79 mutant *irs1* (37) with pEBS7 libraries also produced transformants having improved MMC resistance. However, this resistance was consistently highly unstable among several transformants. By preparing a Hirt extract (38) and transfecting *E. coli*, the correcting cDNA was directly isolated as a plasmid (39). This analysis proved that the cDNA (designated *XRCC2*) was replicating episomally in the hamster cells, although at a very low efficiency (<1 copy/cell). To generate stable transformants, it was necessary to subclone the *XRCC2* cDNA into the pcDNA3 expression vector, which has only a bacterial origin of replication (39).

The third hamster cell mutant that was corrected using a pEBS7 library was CHO UV40 (40). Analysis of primary transformants showed that they were phenotypically stable and that episomal replication of the correcting cDNA was not occurring (N. Liu, personal communication). In this study, one secondary transformant was identified among 2.6×10^8 transfected cells (41). The complementing *XRCC9* cDNA was rescued from this transformant by using polymerase chain reaction (PCR) based on primers specific for the vector sequences (41). In this study, the *XRCC9* mRNA was absent in the UV40 cells, demonstrating that the correcting sequence was not a phenotypic suppressor gene different from that of original mutation (41).

The cloning of *XRCC4*, which corrects CHO XR-1 (42), was done using a complex, elegant strategy in which the complementing function was selected on the basis of V(D)J inversional recombination, which is mediated by the *RAG1* and *RAG2* recombinase genes (43). Selection of the *XRCC4* function was done on the basis of inversion of orientation of the *E. coli gpt* gene within the recombination substrate, which allowed for *gpt* expression from an LTR promoter to confer resistance to mycophenolic acid. A human cDNA expression library in the vector pcDNA1 was linearized and ligated to form concatemers before transfection. Transformed XR-1 cells carrying the V(D)J recombination substrate including *gpt* and expressing the *RAG1* gene were transfected simultaneously with the *RAG2* gene, a cDNA expression library, and the *Puro* dominant marker (for selection of secondary transformants). To rescue the complementing *XRCC4* cDNA, DNA from secondary transformants was digested with *SfiI*, circularized, and transfected into *E. coli*. Kanamycin selection was used to recover *XRCC4* cDNA, which was linked to the *neo* gene present in pcDNA1.

These successful cloning studies using cDNA library transfection of hamster cells argue for attempting to recover additional genes in this manner by using

various hamster cell mutants (44). Some of these mutants may represent functions that are not present in yeast or bacteria (an example is p53), which means that the mammalian sequences cannot be cloned using methods discussed below that are based on homology with known microbial genes.

3.3. Complementation of Microbial Mutants by Human cDNA

In several instances, it has been possible to isolate human cDNA by complementation of bacterial mutants. Two laboratories cloned the *APDG/AAG* cDNA, which encodes a glycosylase that removes 3-methyladenine and 7-methylguanine. In both cases, the approach involved transfecting human cDNA sequences into *E. coli alka tagA* mutants deficient in this activity, and then selecting for resistance to methylating agents (45,46). Surviving clones carried the *APDG/AAG* cDNA. *MGMT* encodes a protein that transfers the methyl group from *O*⁶-methylguanine to a cysteine group on the protein in a noncatalytic manner. The human *MGMT* cDNA was isolated in one study by complementing the *ada* mutant of *E. coli* (47). Yeast mutants have also been used for selecting human cDNA. A yeast temperature-sensitive *cdc9* mutant defective for DNA ligase activity was used to select for a cDNA sequence encoding human *LIG1* (48). This method of complementation across highly divergent species is necessarily restricted to proteins that have highly conserved functions that do not depend on protein interactions. For example, many human and yeast repair protein homologs are not expected to crosscomplement efficiently because of their degree of evolutionary divergence, e.g., ERCC1 and RAD10 (49).

4. Antibody Screening of Bacterial Expression Libraries

Some of the first human repair protein cDNAs were isolated using bacterial expression libraries in which an antibody to a known protein was used as the probe. The λ gt11 libraries were used to isolate cDNAs for uracil DNA glycosylase, apurinic/apyrimidinic endonuclease, polymerase β , and the Ku70 and Ku86 subunits of the Ku autoantigen (which correspond to XRCC6 and XRCC5, respectively) (50). The use of bacterial expression libraries seems to have been superseded by the use of primers based on peptide sequence derived from purified protein.

5. Peptide-Based Degenerate Primers

In many instances, a DNA repair protein (e.g., replication protein A [RPA], DNA ligase I, thymine glycosylase, apurinic endonuclease, 5'-flap endonuclease, and DNA-dependent protein kinase) was purified and characterized before the gene was available. In these cases, peptides derived from the proteins were the starting point for cDNA isolation. Peptide amino acid sequences

were used to design degenerate oligonucleotides, which were sometimes used directly as probes for library screening to recover cDNAs (*hhRAD23B*, *hRPA2*, *DDB2*, *LIG1*, *APE*, *FEN1*, *DNA-PK_{cs}*; cf. **Table 1**). In other studies, degenerate oligonucleotides were used first in polymerase chain reactions (PCRs) having poly(A)⁺ RNA or cDNA as the template. The resulting PCR products were then validated and used to screen cDNA libraries (e.g., *XPC*, *hRPA1*, *hRPA3*, *TDG*, *LIG3*, *hMSH6/GTBP*). The general approach of beginning with peptide sequence for cloning should be valuable in the future, since unknown proteins are encountered as members of complexes containing known repair proteins.

6. Library Screening Probes Based on Homology

During the last several years, it has become possible to isolate numerous human cDNAs based on sequence conservation. Many of the examples are for genes in the pathways of homologous recombination and mismatch repair, in which there is substantial evolutionary conservation (**51,52**). The mammalian proteins in these cases often show significant similarity with proteins identified in chicken, *Drosophila*, yeast, and even bacteria. Thus, in the pathway of homologous recombination, in which *RAD51*, *RAD52*, and *RAD54* appear to be the most essential genes in *S. cerevisiae* for repairing radiation-induced double-strand breaks, human and mouse homologs were readily isolated by preparing primers based on the most conserved regions of these proteins. In each case, PCR products derived by using degenerate oligonucleotide primers were used as probes for library screening.

7. Homology Comparison Combined with Database Analysis

With the recent advent of rapidly expanding databases, including public databases, which are accessible on the Internet, cloning based on homology has further accelerated. Human homologs of microbial genes have been identified by searching the databases for human cDNA sequences, which, when translated in all reading frames, show significant sequence similarity in one of the translations to known microbial sequences. Several mismatch repair gene cDNAs (e.g., *hMLH1*, *hPMS1*, *hPMS2*) were recovered by searching expressed sequence tag (EST) databases of human sequences for homology with bacterial MutL and its yeast homolog. In some cases (**53**), the identified cDNAs contained the complete open reading frames. The I.M.A.G.E. Consortium (**54**) has generated a collection of publicly available human and mouse cDNA clones and sequences, with the aim of encompassing the entire set of expressed human sequences. This collection was used to identify three human homologs of *RAD51* by searching the database for RAD51-like sequences. In each instance, truncated cDNA clones were obtained from the I.M.A.G.E. collection of arrayed clones, and two of these sequences were extended by the GeneTrapperTM (Life

Technologies, Gaithersburg, MD) method to obtain the complete open reading frame. These three human paralogs of *RAD51* were given the designations *RAD51B*, *RAD51C*, and *RAD51D* (**55a,55b**). Similar usage of the I.M.A.G.E. collection of cDNAs resulted in a human (functional) homolog of the *RAD9* in *Schizosaccharomyces pombe* (**56**).

The power of using databases to identify rapidly clones based on sequence similarity is further emphasized by the example of the *hOGG1* gene, whose protein is a glycosylase for 8-hydroxyguanine and also has β -lyase activity. Recently five laboratories reported, almost simultaneously, the cDNA sequence of *hOGG1/hMMH*, which was obtained in each case by identifying a partial clone in a database and then using that clone as a probe for a library screen (**57–60**), or by performing RT-PCR reactions followed by 5'-end extension (**61**) using the rapid amplification of cDNA ends (RACE) procedure (**62**). Similarly, cDNAs for two DNA ligases (*LIG3* and *LIG4*) were identified by screening a database with a peptide sequence that is conserved among human *LIG1* and the ligases of vaccinia virus, *S. cerevisiae*, and *S. pombe* (**63**). cDNA clones that contained the complete open reading frames were obtained by library screening using the initial clones as probes.

8. Positional Mapping Strategies

In several instances involving human genetic disorders related to DNA repair, gene cloning was enabled by performing high-resolution chromosomal mapping of the gene. Both the *ATM* gene of ataxia telangiectasia (**64**) and the *BLM* gene underlying Bloom syndrome (**65**) were mapped to regions of 250–500 kb that were defined by markers. In the case of *BLM*, the genomic localization was done by a novel method termed “somatic crossover point mapping” (**65**). In both studies, immobilized genomic sequences from contigs composed of yeast artificial chromosome (YAC), P1 bacteriophage, and cosmid clones were used to select directly for hybridizing cDNAs (**66**). Enriched cDNAs were recovered by PCR using vector-specific primers for *BLM* cDNA (**65**), or by the retention of biotinylated genomic DNA on streptavidin-coated magnetic beads (**67**) for *ATM* cDNA (**64**). Biotinylation was done using 5'-biotinylated primers to amplify small amounts of digested genomic DNA to which linkers had been ligated (**67**).

Isolation of the *hMSH2* cDNA was facilitated by an approach that involved first analyzing a panel of somatic cell hybrids that contained different portions of chromosome 2, to define a 0.8-Mb interval in which the HNPCC locus resided (**68**). Similarly, using *irs1* radiation hybrids carrying small regions of human chromosome 7 containing the complementing gene, *XRCC2* was localized by marker mapping to a YAC clone that had complementing activity (**69**). The YAC was further subcloned to obtain complementing PAC and cosmid

clones. The demonstrated success of positional mapping must be viewed in terms of its complex, lengthy, and laborious nature.

9. Protein Interaction Methods

9.1. Yeast Two-Hybrid System

One way of discovering new candidate DNA repair genes is to identify a protein that interacts with a known repair protein. The “two-hybrid” genetic system in yeast provides a means of identifying interacting proteins (70). This test is based on expressing in yeast two gene-fusion proteins, one of which contains the protein of interest and the other a representative sequence from a library. If interaction occurs between the target protein and a library sequence, the fusion domains of a transcription factor are brought into juxtaposition, which allows expression of a reporter gene, such as *lacZ*. This method was used to identify *hMRE11*, which encodes a protein that interacted with DNA ligase I (71). *S. cerevisiae MRE11* participates in the repair of double-strand breaks in an end-joining pathway (nonhomologous recombination). Other human repair genes have been used to identify candidate repair genes. For example, *UBL1* (72) and *UBE2I/hUBC9* (73,74) were identified through interactions with *HsRAD51*, but it is not known whether these proteins play any role in DNA repair.

9.2 Identification Based on Protein Interactions in Mammalian Cells

Another approach that deserves more exploitation is the discovery of new repair proteins through characterization of multiprotein complexes. This approach is illustrated by the HHRAD23B protein in NER, which was first found in a complex with XPC when the activity complementing XP-C cells was purified (31). Peptide sequence from HHRAD23B was used to design a probe for library screening. In yeast, the RAD50 protein appears to act in a complex with several other proteins, including XRS-2 (75). Isolation of the equivalent complex in mammalian cells may provide a means of identifying the human counterparts of the yeast proteins (J. Carney, personal communication).

10. Outlook

Among the ~60 entries in **Table 1**, the first gene reported was *p53* followed by *ERCC1* the next year (1984). The pace of gene identification has accelerated, with more than half of the current list reported after 1993. Except for some genes in the NER pathway and positional cloning studies, cDNA cloning has almost always preceded gene isolation. There has been a major shift in cloning strategies for human and mouse genes. With improvement of expression cDNA libraries, functional complementation using genomic DNA has

been replaced by cDNA transfection with notable success in both human and hamster cells. More recently, homology based on known microbial genes, in combination with database analysis, has been widely used. This approach is greatly enhanced by the availability of arrayed cDNA clones in the I.M.A.G.E. EST collection. Once a cDNA is in hand, the human or mouse gene can be obtained quickly from commercial sources that screen large-insert arrayed libraries.

For several of the pathways, cDNAs have been obtained by designing probes based on peptide sequence from purified proteins. This has been a reliable, productive approach. However, some new mammalian genes will not have obvious homologs in lower organisms. As known repair proteins are characterized and antibodies made against them, coimmunoprecipitation of multiprotein complexes in cell extracts offers a means to search for new relevant proteins. Coprecipitated proteins can be used to obtain peptide sequences. These sequences can be used to search databases for matches to translated cDNA or genomic clones to obtain a clone or sequence information rapidly for designing a library probe. The remaining genes to be identified are expected to fall mostly into the recombination and checkpoint pathways.

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Note Added in Proof

The gene that is altered in Nijmegen Breakage Syndrome (NBS1, which encodes a protein designated nibrin) was isolated by both positional cloning (*110a*) and a combination of peptide sequence derived from purified protein combined with database analysis (*110b*).

Table 1

Summary of Cloned Human Genes Involved in DNA Repair and Cellular Response to DNA Damage^a

GENE	PATHWAY AND FUNCTION	ISOLATED FIRST: GENE OR cDNA	CLONING STRATEGY	CLONING REFS.
	Nucleotide Excision Repair			
<i>ERCC1</i>	NER ERCC1-XPF 5'-junction endonuclease	Gene	Genomic DNA transfection-complementation	(11)
<i>ERCC2/XPD</i>	NER 5' → 3' helicase (in TFIIH)	Gene	Genomic DNA transfection-complementation	(12)
<i>ERCC3/XPB</i>	NER 3' → 5' helicase (in TFIIH)	Gene	Genomic DNA transfection-complementation	(19)
<i>ERCC4/XPF</i>	NER ERCC1-XPF 5'-junction endonuclease	Gene; cDNA	Chrom. 16 cosmid library transfection-complementation; homology-based RT-PCR, library screen, database analysis, 5'-RACE	(20,76)
<i>ERCC5/XPG</i>	NER, BER 3'-junction endonuclease removal of oxidative damage	Gene; cDNA	Genomic DNA transfection-complementation; serendipity, database analysis	(21,25,77)
<i>ERCC6/CSB</i>	NER transcription coupling	Gene	Genomic DNA transfection-complementation	(22)

Table 1 continued

<i>ERCC8/CSA</i>	NER transcription coupling	Gene	cDNA transfection- complementation, episomal plasmid rescue	(30)
<i>XPA^b</i>	NER damage recognition	Gene	Genomic DNA transfection- complementation	(18)
<i>XPC</i>	NER damage recognition	cDNA	cDNA transfection- complementation, episomal plasmid rescue; peptide-based RT-PCR, library screen	(29,31)
<i>hhRAD23A</i>	NER complexed with XPC	cDNA	Database analysis and library screening	(31)
<i>hhRAD23B</i>	NER complexed with XPC	cDNA	Co-purification with XPC, peptide- based probe for library screen	(31)
<i>hRPA1</i>	NER, homol. recombination single-strand binding, RPA p70	cDNA	Peptide-based PCR, library screen	(78)
<i>hRPA2</i>	NER, homol. recombination single-strand binding, RPA p32	cDNA	Peptide-based probe, library screen	(79)
<i>hRPA3</i>	NER, homol. recombination single-strand binding, RPA p14	cDNA	Peptide-based PCR, library screen	(80)

Table 1 continued

<i>DDB1</i> ^c	NER XP-E damage-specific DNA binding protein, p127 subunit	cDNA	Peptide-based PCR, library screen; homologous probe (simian) for library screen	(81-83)
<i>DDB2</i>	NER XP-E damage-specific DNA binding protein, p48 subunit	cDNA	Peptide-based probe for library screen	(82)
<i>LIG1</i>	NER/BER DNA ligase	cDNA	Peptide-based probe for library screen, yeast mutant complementation; homology-based PCR and library screen	(48,84)
Base Excision Repair				
<i>TDG</i>	BER thymine glycosylase	cDNA	Peptide-based PCR, cDNA library screen	(85)
<i>UDG</i>	BER uracil glycosylase	cDNA	λ gt11 library screen using antibody	(86,87)
<i>APDG/AAG</i>	BER alkyl purine glycosylase	cDNA	Complementation of <i>alkA tagA E. coli</i>	(45,46)
<i>hMUTY</i>	BER glycosylase for A opposite 8-oxo-Gua	Gene, cDNA	Database analysis, PCR, library screens, 5'-RACE	(88)
<i>hABH</i>	BER (function unknown)	cDNA	Database analysis, library screen	(89)

Table 1 continued

<i>hNTH1</i>	BER thymine glycol, urea glycosylase; AP lyase	cDNA	Serendipity, database analysis, library screen; peptide sequence, database analysis, library screens, 5'-RACE	(90,91)
<i>hOGG1/ hMMH</i>	BER 8-OH-Gua glycosylase, β lyase	cDNA	Database analysis, clone retrieval, library screen; database analysis, RT-PCR, 5'-RACE	(57–61)
<i>HAP1/APE APEX/Ref1</i>	BER abasic site endonuclease	cDNA	λ gt11 library screen using antibody; peptide-based probe for library screen	(92–94)
<i>MGMT</i>	direct lesion reversal methyl guanine methyltransferase	cDNA	Complementation of <i>ada E. coli</i> ; cDNA transfection-complemen- tation, PCR rescue; peptide-based probes for library screen	(47,95,96)
<i>Pol β^d</i>	BER polymerase	cDNA	λ gt11 library screen using antibody	(97)
<i>FEN1</i>	BER 5'-flap endonuclease	cDNA	Peptide fingerprinting and database analysis; homology-based PCR and library screen; peptide-based probe for library screen	(98–100)
<i>XRCC1</i>	BER LIG3 cofactor	gene	Genomic DNA transfection- complementation	(23)
<i>LIG3^e</i>	BER DNA ligase	cDNA	Peptide-based sequence comparison with databases, library screen; peptide-based PCR, library screen	(63,101)

Table 1 continued

	Homologous Recombinational Repair			
<i>HsRAD51/ RAD51A</i>	Homol. recombination essential for viability	cDNA	Homology-based PCR, library screen	(102)
<i>HsRAD52</i>	Homol. recombination	cDNA	Homology-based PCR, library screen	(103,104)
<i>HsRAD54</i>	Homol. recombination	cDNA	Homology-based PCR, library screen	(105)
<i>XRCC2</i>	Homol. recombination	cDNA; gene	cDNA transfection- complementation; positional mapping, direct cDNA selection	(39,69,69a)
<i>XRCC3</i>	Homol. recombination	cDNA	cDNA transfection- complementation	(36,39)
<i>RAD51B/ R51H2/hREC2^f</i>	Homol. recombination	cDNA	Homology, database analysis and GeneTrapper™	(55,106,106a)
<i>RAD51C</i>	Homol. recombination	cDNA	Homology, database analysis and GeneTrapper™	(55a)
<i>RAD51D/R51H3</i>	Homol. recombination	cDNA	Homology, database analysis	(55b,106a)
	Nonhomologous Recombinational Repair			
<i>XRCC4</i>	nonhomol. rec., V(D)J rejoining LIG4 cofactor	cDNA	cDNA transfection- complementation	(43)

Table 1 continued

<i>XRCC5/ Ku80</i>	Nonhomol. rec., V(D)J rejoining, scaffold protein?	cDNA	λ gt11 library screen using antibody	(107,108)
<i>XRCC6/ Ku70</i>	Nonhomol. rec., V(D)J rejoining, scaffold protein?	cDNA	λ gt11 library screen using antibody	(109)
<i>XRCC7/ DNA-PK_{cs}</i>	Nonhomol. rec., V(D)J rejoining DNA-depend. protein kinase	cDNA	Peptide-based probe for library screen, secondary screens	(110)
<i>LIG4</i>	Nonhomol. rec., V(D)J rejoining DNA ligase	cDNA	Peptide-based sequence comparison with databases; library screen	(63)
<i>HsRAD50</i>	Nonhomol. & homologous rec., complex with hMRE11-NBS1	gene, cDNA	Direct cDNA selection using YACs, serendipity, 5'-RACE	(74)
<i>hMRE11</i>	Nonhomol. & homologous rec., complex with HsRad50-NBS1	cDNA	Two-hybrid screen with DNA LIG1	(70)
<i>NBS1</i>	Nonhomol. & homologous rec., complex with HsRad50-hMre11	Gene;cDN A	Positional cloning; peptide-based probe for library screenings	(110a,110b)
Mismatch Repair				
<i>hMSH2</i>	Mismatch	cDNA; gene/cDNA	Homology based PCR, library screen; chrom. position, homology- based PCR, library screen	(68,52)
<i>hMSH3</i>	Mismatch	cDNA	Serendipity	(111)

Table 1 continued

<i>hMSH6/</i> <i>GTBP</i>	Mismatch	cDNA	Peptide-based RT-PCR, library screen, RACE	(112,113)
<i>hMLH1</i>	Mismatch	cDNA	Homology, database analysis	(114)
<i>hPMS1</i>	Mismatch	cDNA	Homology, database analysis	(53)
<i>hPMS2</i>	Mismatch	cDNA	Homology, database analysis	(53)
<i>hPMS3-11</i>	Mismatch	cDNAs, genes	Homology based RT-PCR, cDNA and cosmid library screens	(115)
Miscellaneous				
<i>PARP/</i> <i>ADPRT</i>	Regulation of repair? nick sensor	cDNA	λ gt11 library screen using antibody, pcD library screen	(116)
<i>p53</i>	Cell cycle checkpoint(s)	cDNA	cDNA probe prepared from immunoprecipitated polysomal mRNA, library screen	(117)
<i>ATM</i>	Cell cycle checkpoint(s)	Gene	Chromosome position, direct cDNA selection, sequence extension	(64)
<i>hHR21^{Sp}</i>	G ₂ checkpoint?	cDNA	Homology, database analysis, and library screen	(118)
<i>hRAD9^{Sp}</i>	G ₂ checkpoint?	cDNA	Homology, database analysis, and library screen	(56)

Table 1 continued

<i>XRCC9</i>	Checkpoint or postreplication repair?	cDNA	cDNA transfection-complementation, rescue by PCR using vector primers	(41)
<i>BLM</i>	DNA replication?	Gene	chromosome position, direct cDNA selection, sequence extension	(65)
<i>FAA</i>	DNA damage? ^g Reduction of reactive oxygen intermediates	cDNA, gene	cDNA transfection-complementation, episomal plasmid rescue; chromosome position, direct cDNA selection, sequence extension	(32;119)
<i>FAC</i>	DNA damage? ^g Reduction of reactive oxygen intermediates	cDNA	cDNA transfection-complementation, episomal plasmid rescue	(33)

^a Not included in this table are: genes encoding proteins involved in cellular checkpoint control in response to DNA damage that are activated by p53 (e.g. p16^{INK4a} and p21^{CIP1/WAF1}), polymerases such as Pol ϵ (which is apparently involved in NER), polymerase accessory factors such as PCNA and RFC, and additional components of transcription factor TFIIH, some of which have been cloned. Semicolons separate studies using different cloning methods. Abbreviations: RT-PCR (reverse transcriptase-polymerase chain reaction); RACE (rapid amplification of cDNA ends).

^b The mouse gene was cloned before the human.

^c The simian cDNA was cloned before the full-length human.

^d The rat cDNA was cloned before the human.

^e There does not appear to be a distinct gene that encodes DNA ligase II, which is likely to be a processed form of the ligase III protein (A. Tomkinson, personal communication).

^f The designation *hREC2* may not be justified in view of the very weak similarity of the encoded protein with the Rec2 protein.

^g The defect in Fanconi anemia cell lines, rather than affecting repair, may involve an excess of DNA damage, both endogenous as well as that from crosslinking agents, owing to increased levels of reactive oxygen intermediates (120).

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Novel Complementation Assays for DNA Repair-Deficient Cells

Transient and Stable Expression of DNA Repair Genes

Lin Zeng, Alain Sarasin, and Mauro Mezzina

1. Introduction

Xeroderma pigmentosum (XP), trichothiodystrophy (TTD), and Cockayne syndrome (CS) are human genetic diseases associated with defects in nucleotide excision repair (NER). Each disease is subdivided into multiple complementation groups. Identification of the genetic group to which a patient belongs is relevant for both clinical and basic research aims: (1) to study relationships between genotype and phenotype, in order to understand the different clinical symptoms and, eventually, to envisage clinical trials (gene therapy); and (2) to elucidate DNA repair mechanisms in mammalian cells (*1*). Since the first characterization of a DNA repair defect in XP cells, measured by the uptake of ^3H -thymidine during unscheduled DNA synthesis (UDS), assignment of a patient to a complementation group has been achieved by using the somatic cell fusion procedure followed by analysis of UDS or recovery of RNA synthesis (RRS) after ultraviolet (UV) irradiation (*2,3*).

The procedure involves crossfusion of different cell lines, A and B, of known and unknown groups (**Fig. 1**). If normal function is restored in the heterokaryon, i.e., if complementation occurs, then by definition, the genetic groups to which cells A and B belong are different. If no complementation occurs, then cells A and B are said to belong to the same genetic group. This technique has permitted the identification of multiple genetic groups of rodent and human cells presenting a phenotype of reduced NER capacity. However, owing to the large number of crossfusions necessary to test all possible complementation groups, this procedure is time-consuming and tedious to perform. Furthermore,

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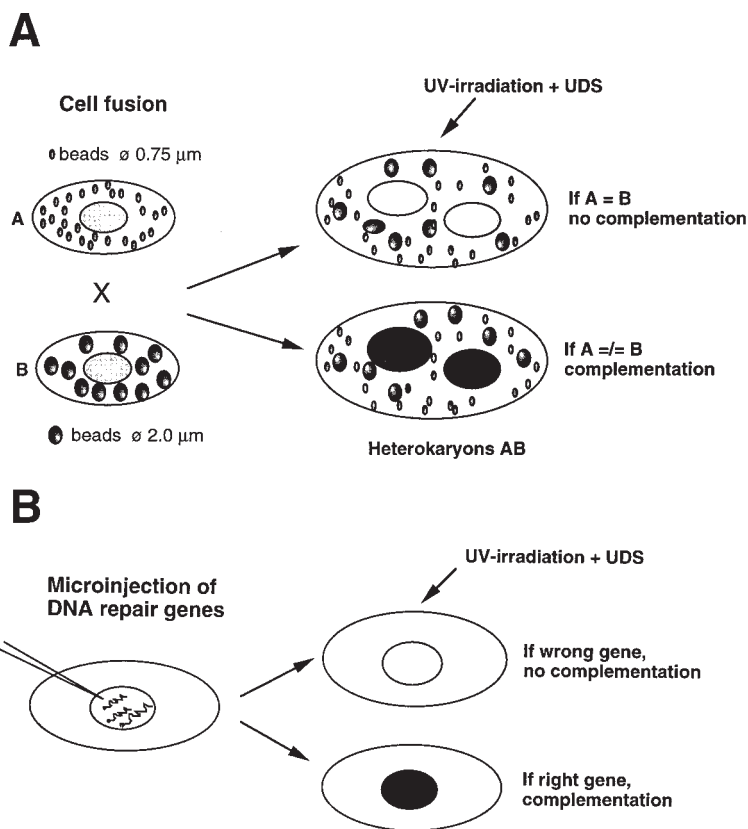


Fig. 1. **(A)** Cell fusion procedure. Beads of two sizes (0.75 and 2.0 μ m in diameter) are used as markers to recognize the fused heterokaryons from homokaryons. Complementation following UV irradiation (represented by dark nuclei) indicates that the two cell lines are in different genetic groups ($A \neq B$). If there is no complementation, the two cell lines are considered to belong to the same complementation group ($A = B$). **(B)** Microinjection of a repair cDNA into a DNA repair-deficient cell. Following UV irradiation, correction of the reduced UDS phenotype indicates that the cell belongs to the genetic group of the injected gene (right gene); if no complementation occurs, the injected gene (wrong gene) is not involved in the DNA repair defect.

when DNA repair efficiency is only slightly reduced, resulting in relatively high levels of UDS, as in some XP and TTD patients, the results are difficult to interpret, even after repeated experiments. An attempt to speed up this procedure (4) proved unsuccessful.

The cloning of human DNA repair genes during the past 10 years (*see* Chap. 7) has opened up new, more direct approaches for determining the genetic group

to which a DNA repair-deficient cell line belongs. The availability of DNA repair cDNAs cloned into appropriate expression vectors has provided the opportunity to establish novel complementation assays after the introduction of these genes into repair-deficient cells. The first technique to be developed was the microinjection procedure (**Fig. 1B**). This involves simply injecting the cloned cDNA into the mutant cell and looking for restoration of normal UDS activity. Although this procedure is simpler and faster than the cell fusion assay, it still involves considerable technical expertise, even when the microinjection system is equipped with sophisticated computer-assisted devices. Moreover, as with somatic cell fusion, microinjection assays are not sufficiently sensitive to be able to detect moderately reduced UDS levels. This chapter details two procedures for assigning a new mutant to a specific complementation group, both based on the introduction and expression of DNA repair cDNAs, followed by analysis of the phenotypic correction of recipient cells.

1.1. Cotransfection of Repair-Deficient Cells with Expression Plasmids Carrying Wild-Type DNA Repair cDNAs and UV-Irradiated Plasmids Carrying Reporter Genes

This procedure (3) is based on the observation that all NER-defective cells are unable to reactivate the expression of UV-irradiated plasmid harboring chloramphenicol acetyl transferase (CAT) or luciferase (L) genes whose expression is under the control of the LTR promoter of Rous Sarcoma Virus (RSV). In this system, unrepaired UV photoproducts block the transcription of the reporter genes. This approach has been extensively used to characterize UV-sensitive mammalian cell lines (5) and to confirm clinical diagnosis in patients with suspected DNA repair syndromes (6). In cells presenting defects in the removal of DNA photoproducts from both transcribed and non-transcribed strands, such as XP cells (7), from only transcribed strands, such as CS cells (8), or from only untranscribed strands, such as XP-C cells (9), the reactivation levels are significantly reduced compared to those of wild-type cells. This implies that this method is sufficiently sensitive to be able to detect defective NER irrespective of the location of the DNA lesion. However, when these mutants are cotransfected with plasmid harboring the gene specific for their complementation group, the extent of reactivation of UV-irradiated reporter plasmids is indistinguishable from that of wild-type cells (**Fig. 2**). The efficacy of this procedure has been demonstrated using both pRSVCAT and pRSVL plasmids in mouse and immortalized human cell lines (10).

Furthermore, because of the much higher sensitivity of the luciferase system over the CAT assay (11), the former can be efficiently used with diploid human fibroblasts obtained from skin biopsies at early passages (up to 10–15). Here, the procedure using the pRSVL reporter system is described.

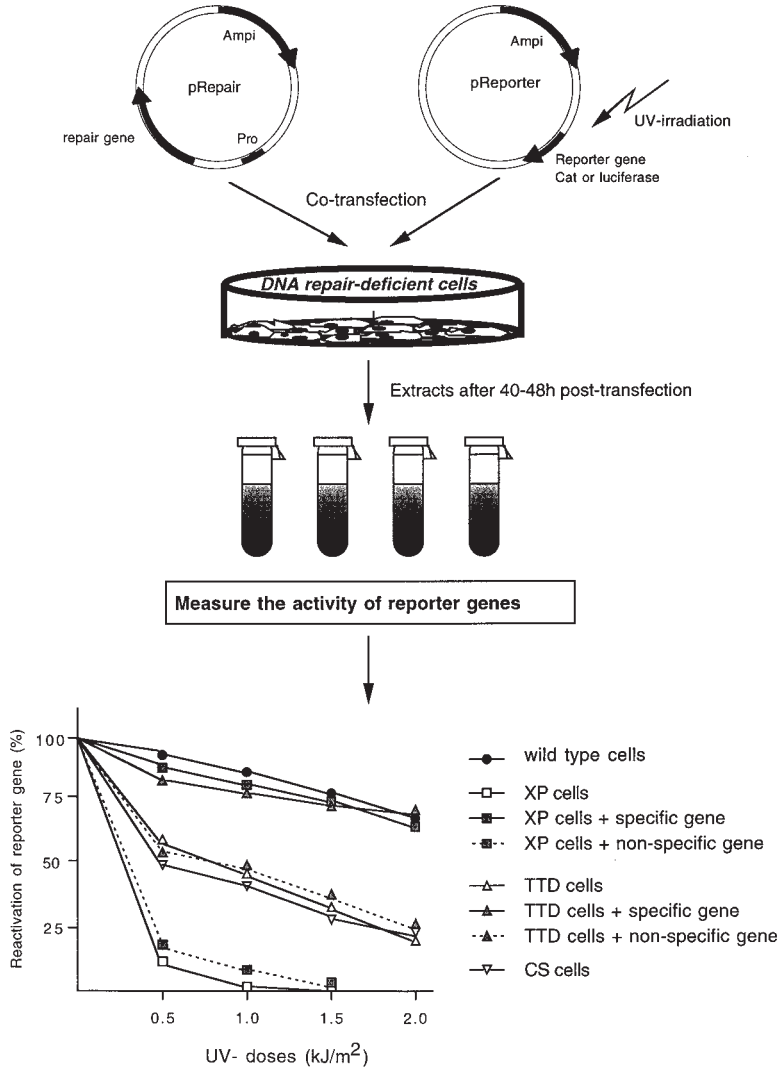


Fig. 2. Assay of reactivation of reporter genes. Cotransfection of expression plasmids carrying a DNA repair cDNA (pRepair) and UV-irradiated plasmid carrying a reporter gene (pReporter) is performed for repair-deficient cells as described in the text. The activity of reporter genes, normalized to protein content and expressed as a percentage of reactivation rates, is the amount of reporter activity of cell extracts transfected with irradiated vs unirradiated plasmid. The curves show the expected result. The reactivation of reporter gene occurs only when cells are transfected with pRepair plasmid carrying the specific gene, since the activity of reporter gene is restored to that of wild-type cells. Therefore, cells belong to the genetic group of the transfected gene.

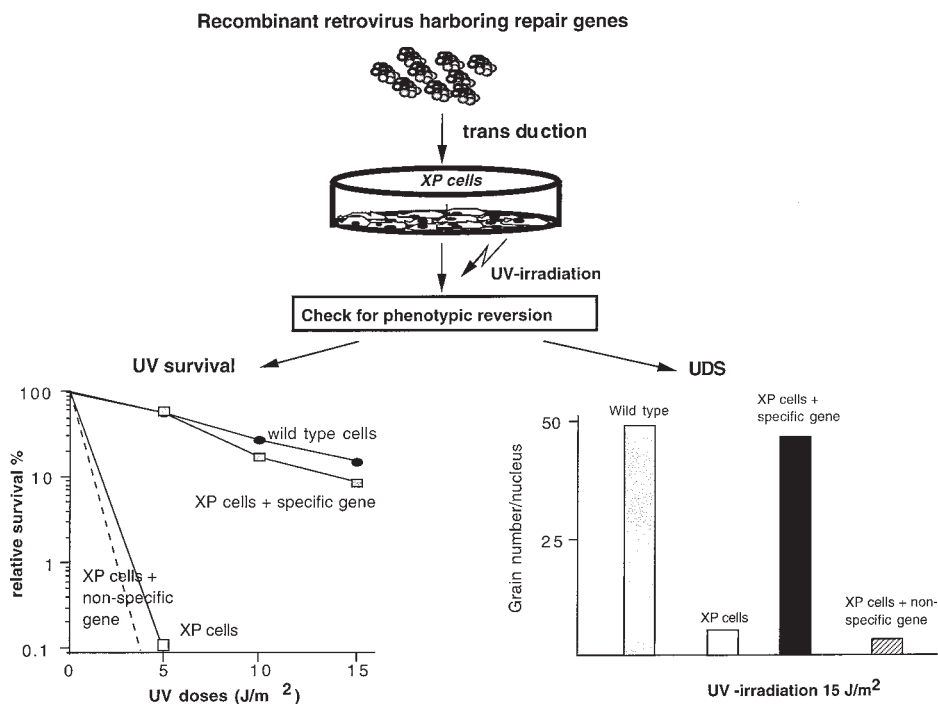


Fig. 3. Transduction assay with recombinant retrovirus. DNA repair-deficient cells, transduced with retroviruses harboring DNA repair genes are checked for phenotypic reversion by either UV survival or UDS assay. Only cells transduced by the specific gene display the corrected phenotype by both assays.

1.2. Retrovirus-Mediated Transduction of DNA Repair Genes into Cells

Several retroviral vectors have recently been constructed to deliver genes of interest into human cells and tissues, with the aim of establishing gene therapy protocols. The first retroviral construction carrying a DNA repair gene, LXPDSN, comprises the LXS vector, which is derived from Moloney murine leukemia virus (12), and an *XPB* cDNA (13). Three further constructions, LXPASN, LXPBSN, and LXPCSN, which carry the *XPA*, *XPB*, and *XPC* cDNAs, respectively, have also been made in an effort to develop a gene therapy protocol for skin cancers in XP. Recent results have revealed these vectors to be highly efficient tools for transferring DNA repair genes into repair-deficient human fibroblasts, both diploid or immortalized, and to fully correct their phenotype (14). In principle, therefore, these constructions should also be useful for routine complementation testing of cell lines derived from

XP patients. The general procedure of the assay is outlined in **Fig. 3**. The retroviral vector carrying the gene of interest is transfected into packaging cell lines ψ Crip or ψ CRE, which produce viral particles able to infect recipient cells. The neomycin (G418) resistance gene contained in the vector is used as a selectable marker (**15**). Infected fibroblasts are selected by G418 and then analyzed for phenotypic correction. **Fig. 3** shows that the transgene is able to correct the DNA repair-deficient phenotype fully, since both UV survival and UDS are corrected only in those cells transduced with the specific DNA repair gene for that complementation group. The main advantages of this approach are: (1) stable expression of the foreign transgene can be achieved in all types of cell lines (transformed or diploid fibroblasts); and (2) the stable integration of only one or two copies of the transgene in recipient cells avoids possible phenotypic changes owing to overexpression of the cDNA, such as may occur by either microinjection or transfection.

2. Materials

2.1. Cell Culture (see Note 1)

1. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with glutamine containing 100 U/mL penicillin G, 100 μ g/mL of streptomycin, and 2.5 μ g/mL of fungizone (Gibco BRL, Life Technologie, Cergu Pontoise, France).
2. Eagle's Minimal Essential Medium (MEM) supplemented with glutamine containing 100 U/mL penicillin G, 100 μ g/mL of streptomycin, and 2.5 μ g/mL of fungizone (Gibco BRL).
3. Fetal bovine serum (Dominique Dutscher Strasbourg, France).
4. Bovine calf serum (BCS) (HyClone, Erembodegem Aalst, Belgium).
5. Trypsin/EDTA solution (Gibco BRL).
6. Phosphate-buffered saline (PBS), pH 7.4: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 (Gibco BRL).
7. 3-, 6-, and 10-cm Tissue-culture dishes, 25- and 75-cm² flasks and six-well culture plates.
8. 37°C, 5% and 10% CO₂ humidified incubators.

2.2. Preparation of Plasmid DNA

1. LB medium containing ampicillin 50–100 μ g/mL.
2. Glucose/Tris/EDTA solution (Sol I): 50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10 mg/mL lysozyme.
3. NaOH/SDS solution (Sol II): 0.2 N NaOH, 1% sodium dodecylsulfate (SDS). Make fresh.
4. Potassium acetate solution (Sol III): 3 M K-acetate, 5M acetic acid, prepared by mixing 120 mL 5 M potassium acetate, 23 mL glacial acetic acid, and 57 mL H₂O. The pH of the resulting solution is 4.8.
5. Isopropanol and ethanol (Merk, Nogent-sur Marne, France).

6. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
7. Cesium chloride solution: add 40 g of CsCl (Gibco or Sigma, St. Quentin Fallavier, France) to 40 mL of TE and adjust the refractive index to $\rho = 1.392$.
8. 10 mg/mL Ethidium bromide (Sigma) solution. **Caution:** Ethidium bromide is a powerful mutagen and is toxic. Gloves should be worn when working with solutions that contain this dye. It should be discarded as are other toxic compounds.
9. 20X SSC-saturated isopropanol. 20X SSC is 3 M NaCl, 0.3 M Na₃ citrate. Adjust to pH 7.0 with 1 N HCl.

2.3. Luciferase Assay

1. Luminometer (Nucleotimetre 107, C.L.V. - InterBio®, Berthold, Germany).
2. Plastic minivials.
3. PBS. *See item 6, Subheading 2.1.*
4. Cell lysis buffer (Promega): 25 mM Tris-phosphate pH 7.8, 2 mM dithiothreitol (DTT), 2 mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA).
5. Luciferase assay buffer (Promega, Charbonnieres, France): 20 mM Tricine, 1.07 mM MgCO₃, 2.57 mM MgSO₄, 0.1 mM EDTA, 33.3 mM DTT, 0.27 mM Coenzyme A (CoA), 0.47 mM luciferin, 0.53 mM ATP, final pH 7.8.
6. Bradford buffer for colorimetric assay of protein quantity (Gibco BRL): 0.01% Coomassie brilliant blue G-250 (Sigma), 4.7% ethanol, and 8.5% phosphoric acid or Protein Assay kit (Bio-Rad, Ivry sur Seine, France).

2.4. Production of Retrovirus and Transduction of Cells

1. Plasmid DNA of retroviral vector (purified by centrifugation in a cesium chloride gradient containing ethidium bromide).
2. 2.5 M CaCl₂.
3. 2X HEPES-buffered saline (2X HBS) solution: 280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄, 12 mM glucose, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), titrate to pH 7.05 with NaOH.
4. 4 mg/mL Polybrene (Sigma).
5. Neomycin (G418) at 50 mg/mL (Gibco BRL).
6. Cloning rings.

2.5. Measurements of DNA Repair: UV Survival and UDS

1. Germicidal UV lamp at 254 nm.
2. UV light dosimeter.
3. Fetal bovine serum dialyzed with PBS and sterilized by filtration.
4. 1 mM fluorodeoxyuridine.
5. ³H-thymidine (SA of 50 Ci/mmol).
6. 1 M cold thymidine.
7. 5% Trichloroacetic acid (TCA) at 4°C.
8. Methanol.
9. Ethanol.
10. PBS. *See item 6, Subheading 2.1.*

11. Glass microscope slides ($2.5 \times 7.5 \text{ cm}^2$) and sterile glass cover slips ($2 \times 2 \text{ cm}^2$).
12. Mounting medium for microscopic preparations: Eukitt (Freiburg, Germany).
13. Kodak NTB-2 emulsion.
14. Staining solutions: 2.5 g crystal violet dissolved in 900 mL of methanol and 100 mL of formaldehyde, or 4% Giemsa solution (Merk) in PBS.

3. Methods

3.1. Large-Scale Plasmid DNA Preparation

1. Grow bacterial cultures (500 mL) overnight at 37°C to stationary phase and then centrifuge at 6000 rpm (6000g in a GSA, GS-3, or SLA-3000 rotor) for 20 min. Wash the pellets with 10 mL of PBS, transfer to 50-mL (nominal capacity) Nalgene tubes, and centrifuge at 6000g for 20 min. Discard the supernatant.
2. Resuspend the pellets in 5 mL of Sol I, vortex vigorously, and incubate for 15–60 min at room temperature.
3. Add 10 mL of Sol II to the first lysate, and mix by inverting the tubes several times. Do not vortex. Shake the tubes gently on a shaker for 20 min, and then place the tubes on ice for 10 min.
4. Add 7.5 mL of Sol III. Mix by inversion and put the tubes on ice for 10 min. A white precipitate can be seen.
5. Spin at 16,000 rpm for 40 min in an SS-34 rotor (31,000g) at 4°C . A shorter spin may result in some chromosomal contamination.
6. Collect the supernatants in fresh Nalgene tubes.
7. Add 0.6 vol of isopropanol, mix by inversion, and let stand at room temperature for at least 30 min. The tubes can be kept at 4°C overnight.
8. Spin at 12,000 rpm in an SS-34 rotor (17,000g) for 30 min at 15°C .
9. Discard the supernatants, and wash the pellets with 70% ethanol.
10. Spin at 12,000 rpm (17,000g) for 15 min.
11. Dry the pellets under vacuum for at least 10 min.
12. Add 2 mL of TE. Swirl gently for at least 20 min to dissolve the DNA (sometimes it may take longer).
13. Put 3.5 g of CsCl into the tubes, add 1.5 mL of TE buffer, and 280 μL of ethidium bromide solution (10 g/mL). Mix by inversion until the CsCl is dissolved.
14. Spin the solution at 10,000 rpm (12,000g) for 20 min at 20°C , and recover the clear, red supernatant.
15. Check that the refractive index $\rho = 1.392$ with the refractometer. Adjust by adding CsCl (if lower) or TE buffer (if higher) (*see Note 2*).
16. Transfer the solution into a Quick-Seal centrifuge tube, and adjust the volume up to the top (5 mL) with CsCl solution ($\rho = 1.392$).
17. Centrifuge in a VTi 90 rotor (Beckman) at 60,000 rpm (287,000g) for 16 h at 19°C .
18. Gently remove the tubes. Two parallel bands (the upper is nicked circular or linear DNA, and the lower is closed circular plasmid DNA), and a vertical band attached to the tube wall (protein) can be found. Recover the closed circular plasmid DNA (lower band) with a sterile syringe needle and put in a sterile 15-mL tube.
19. Add CsCl solution up to 5 mL, and check again that $\rho = 1.392$ with the refractometer.

20. Transfer the solution into a new Quick-Seal centrifuge tube and centrifuge at 75,000 rpm (448,000g) for 4 h as in **step 17**.
21. Recover the DNA as in **step 18**. It should appear as a single major band. Transfer the DNA to a 15-mL conical tube.
22. Remove the ethidium bromide from the DNA with NaCl-saturated isopropanol. Mix the two phases by vortexing, and wait 1–2 min. When the two phases are separated, transfer the lower, aqueous phase to a clean tube using a Pasteur pipet. Repeat the extraction four to six times until all the pink color disappears from both the aqueous and organic phases.
23. Dialyze the DNA solution against several changes of TE buffer.
24. Measure the OD₂₆₀ of the final solution of DNA and calculate the concentration of DNA.
25. Sterilize the DNA solution by vigorously mixing it with 1 vol of chloroform-isoamyl alcohol (24:1) followed by three extractions with 1 vol of ether in a glass tube or a 15-mL conical polypropylene tube. When the two phases are separated, using a Pasteur pipet, transfer the lower, aqueous phase to a clean tube. Repeat this step three times and keep the final tube uncapped inside a culture hood overnight (*see Note 3*).

3.2. Calcium Phosphate-Mediated DNA Transfection

1. Prepare DNA/CaCl₂ solution: Dilute 1–5 µg of plasmid DNA (dissolved in TE) with sterile water to 225 µL, and add 25 µL of 2.5 M CaCl₂ (final concentration 250 mM).
2. Place 250 µL of 2X HBS in a sterile transparent tube.
3. Add dropwise with gentle mixing 250 µL of DNA/CaCl₂ solution to 250 µL of 2X HBS and then incubate the mixture for 30 min at room temperature. A fine precipitate should be produced that can be visualized using a microscope.
4. Mix freshly trypsinized cells (3–5 × 10⁵) with the DNA precipitate suspension, seed six-well culture plates or 10-cm dishes with 3 or 10 mL of culture medium, respectively, and incubate overnight (12–16 h) in a 5% CO₂ incubator at 37°C. For transfection of monolayer cells, add 0.5 mL of the calcium phosphate–DNA coprecipitate to 5 mL of medium in a 6-cm dish (*see Note 4*).

3.3. Luciferase Assay

1. Wash the cells twice with prechilled PBS.
2. Trypsinize the transfected cells, or add 100–250 mL of cell lysis buffer to monolayer cells and then scrape the cells with a rubber policeman. Collect the cells in a 1.5-mL Eppendorf tube (*see Note 5*).
3. Add 250 µL of cell lysis buffer to the trypsinized cells, and incubate for 2 min at room temperature.
4. Spin in a microcentrifuge at maximum speed (~12,000g) for 1 min.
5. Recover the supernatant, and use 10–50 µL of extract for the luciferase activity assay.
6. Add 50 µL of the luciferase assay buffer to the extract and mix.

7. Measure the luciferase activity in a luminometer apparatus in a peak or integral mode, and normalize for protein amount in the extracts as quantified by the Bradford's colorimetric assay (e.g., Bio-Rad).
8. Calculate the rate of reactivation of UV-irradiated reporter plasmid as the specific activity of luciferase in cells transfected with UV-irradiated/unirradiated plasmid.

3.4. Virus Production by Transient Transfection of Packaging Cells

1. Day 1: Seed the retrovirus-packaging cells (PE501, PA317, ψ Crip or ψ CRE) at 5×10^5 cells/6-cm dish.
2. Day 2: Replace the culture medium with 4 mL of fresh medium and transfect the cells with vector plasmid DNA as described in **Subheading 3.2**.
3. Day 3: Replace the medium with 4 mL of fresh medium.
4. Day 4: Collect the virus-containing medium, and filter through a 0.45- μ m filter to remove cells and debris. This process can be repeated three or four times at 12-h intervals from the same dish of cells. Retrovirus-containing medium can be used to transduce the target cells or kept at -70°C for long-term storage (*see Note 6*).

3.5. Generation of Stable Vector-Producing Cell Lines

This method has been modified according to (16).

1. Day 1: Seed ψ CRE (Ecotropic retrovirus-packaging cell) at 5×10^5 cells/6-cm dish.
2. Day 2: Replace the culture medium, with 4 mL of fresh medium and transfect the cells with vector plasmid DNA by calcium phosphate precipitation (*see Subheading 3.2*).
3. Day 3: Replace the culture medium with 4 mL of fresh medium. Seed ψ CRIP (Amphotropic retrovirus-packaging cell) at 5×10^5 cells/6-cm dish. Use 2 dishes for each dish of transfected ψ CRE.
4. Day 4: Replace the medium in the ψ CRIP dishes with medium containing 4 $\mu\text{g/mL}$ Polybrene. Remove 3 mL of virus-containing medium from each dish of transfected ψ CRE (leave 1 mL to keep the cells from drying out until they are trypsinized), and filter the medium as in step 4 of **Subheading 3.4**. From each dish of transfected ψ CRE, use 1 mL of virus-containing medium to infect one dish of ψ CRIP, and add 10 μL to another dish of ψ CRIP. Trypsinize and seed the ψ CRE at a 1:20 dilution into 6-cm dishes containing medium with 1 mg/mL G418 for vector carrying the *neo* gene, 4 mM histidinol for vector carrying the *hisD* gene, and 0.4 mg/mL hygromycin B for vector carrying the *hph* gene. These dishes are stained and evaluated for colony formation after 5 d of selection as a measure of the efficiency of DNA transfection.
5. Day 5: Trypsinize the infected ψ CRIP, and seed the cells at 9:10 and 1:10 dilution into 10-cm dishes containing 10 mL medium supplemented with 0.5–1.0 mg/mL G418. After G418-resistant colony formation (5–10 d of selection), isolate clones from dishes containing small numbers of colonies by using cloning rings.

3.6. Determination of Virus Titers

1. Day 1: Seed recipient NIH 3T3 cells at 5×10^5 cells/6-cm dish.
2. Day 2: Replace the medium with medium containing 4 $\mu\text{g/mL}$ Polybrene, and add various dilutions of test virus.
3. Day 3: Trypsinize and dilute the cells 1:20 into medium containing 0.5–1.0 mg/mL G418 for vector carrying the *neo* gene. The concentration may be adjusted depending on the cell lines. Keep in culture 10–14 d.
4. Days 10–14: Stain and count colonies. Virus titer in colony-forming units per milliliter (*cfu/mL*) is calculated by dividing the number of colonies by the volume (in milliliters) of virus used for infection and multiplying by 20 to correct for the 1:20 cell dilution.

3.7. Transduction of Cells with Retrovirus

1. Day 1: Seed the virus-producing cells (for example, ψCrip or PA370) at 1×10^6 /10-cm dish or each 75-cm² flask.
2. Day 2: Seed the cells to be infected (e.g., fibroblasts, epithelial cells, lymphocytes, and so forth) at low density in 25-cm² flasks or 6-cm dishes. Twelve to 24 h before transduction, replace the medium of the virus-producing cells with 4 mL of fresh medium to collect retrovirus.
3. Day 3: Collect the virus-containing medium, and filter through a 0.45- μm filter.
4. Aspirate the medium from the cells to be transduced and replace with virus-containing medium at several dilutions. Then add Polybrene at 4 $\mu\text{g/mL}$.
5. Change the medium with fresh medium 48–72 h later.
6. When the cells grow, add neomycin at 0.5–1.0 mg/mL (other drugs may be used depending on the selectable genes carried by the vectors) to select the transduced cells (see Note 7).

3.8. UV Survival Test

1. Day 1: Seed the primary fibroblasts in 10-cm culture dishes in complete medium supplemented with 20% of fetal bovine serum (10% serum for transformed cells). (See Note 8.)
2. Day 2: Replace the medium with fresh complete medium.
3. Day 3: Remove the medium, and irradiate the cells with UV-C at several doses, for example, from 2–20 J/m². Add the fresh medium. Keep in culture until 14th d.
4. Day 14: Aspirate the medium, and wash the cells with PBS. Stain the cells with staining solutions (crystal violet or Giemsa) for 30 min. UV survival rate is calculated by counting the number of cell colonies according to the cell number before UV irradiation, as the percentage of UV irradiated cells over unirradiated cells.

3.9. Unscheduled DNA Synthesis

1. Day 1: Seed fibroblasts in 3-cm dishes with a sterile cover slip in complete F-10 medium supplemented with 15% fetal bovine serum.

2. Day 2: Replace the medium with the complete F-10 medium supplemented with 1% dialyzed fetal bovine serum.
3. Day 3: Aspirate the medium, and irradiate the cells with UV. Add F-10 medium with 1% dialyzed fetal bovine serum, 1 μ M fluorodeoxyuridine, and 10 μ Ci/mL of ^{3} H-thymidine. Incubate at 37°C for 3 h (*see Note 9*).
4. Change the medium with F-10 medium containing 1% dialyzed fetal bovine serum, 1 μ M of fluorodeoxyuridine, and 1 μ M of cold thymidine. Incubate at 37°C for 1 h.
5. Wash the cells three times with cold PBS.
6. Fix the cells with methanol for 10 min.
7. Fix the nucleic acid twice in 5% trichloroacetic acid on ice, each for 15 min.
8. Wash the cells once with 70% ethanol and twice with absolute ethanol. Dry the coverslip in air.
9. Mount the cover slip on a glass microscope slide with mounting medium. Position the cover slip so that the cellular side is up. Dry overnight.

Steps 10–13 should be carried out in a dark room.

10. Prewarm Kodak NTB-2 emulsion to 42°C.
11. Plunge the slide with cover slip into the emulsion for 1 min, and air-dry for 4 h.
12. Put the slides in a box, wrap it with aluminum foil, and keep at 4°C for 5 d.
13. Develop the slides with Kodak D19 at 13°C for 4 min, and fix for 10 min.
14. Gently wash the slides in tap water for 5 min.
15. Stain the cells with Giemsa solution for 15 min. Wash and dry the slides in air.
16. Mount another cover slip on the cells with Eukitt mounting medium.
17. Using a microscope, count the grain number in each nucleus. DNA repair synthesis rate is expressed by mean grain number of at least 30 nuclei.

4. Notes

1. NIH3T3 fibroblasts and retrovirus packaging cell lines ψ CRE and ψ CRIP are grown at 37°C in 10% CO₂ in DMEM medium supplemented with 10% bovine calf serum and antibiotics.
2. We recommend using DNA obtained in large-scale by alkaline lysis of bacteria followed by double cesium chloride gradient centrifugation. DNA kept in sterile conditions at 4°C will last for several years without degradation and loss of transfection efficiency. One of the most important steps is adjustment of the refractive index of the DNA/cesium chloride solution to exactly $n = 1.392$ before centrifugation. Also, it is best to use a fresh preparation of ethidium bromide solution (10 mg/ml).
3. Glass tubes or conical polypropylene tubes should be used because they are ether-resistant. Keep the tube open in a culture hood overnight to evaporate residual ether, which removes organic solvents by extraction. This is important because traces of the organic solvents may decrease the transfection efficiency.
4. Cells can be subjected to glycerol or DMSO shock (15% in PBS), for 30 s to 2 h, followed by three washes with PBS, and then incubated for a further 40–48 h in fresh complete growth medium. Although a glycerol or DMSO shock may

- increase the efficiency of transformation in some cell lines, it may be toxic in other cell lines. Therefore, it is recommended that this parameter be checked for each cell line to be transfected.
5. Cell pellets can be kept at -20°C for several weeks or months, and cell extracts kept at room temperature for several hours, without losing enzymatic activity.
 6. Virus can be generated from the plasmid of retroviral vectors by transient transfection of packaging cells ψCrip or ψCRE or as a first step in generating stable vector-producing cell lines. We use calcium phosphate coprecipitation for introduction of vector DNA into cells.
 7. Many cell types can be infected by direct exposure to virus. The addition of $4\text{ }\mu\text{g/mL}$ Polybrene or protamine sulfate during infection facilitates infection. These compounds are positively charged (polycationic), and presumably act by neutralizing negative charges present on the surface of cells and virions to allow better binding. Always centrifuge or filter virus before transduction to avoid contamination of the target cells with packaging cells that may be present in virus-containing medium. In our experience, filtration is a better method. Because of the apparent need for cell division during retrovirus infection, cells should be seeded at low density to allow cell division during infection. Plating at low density also helps to reduce cell fusion that can occur in cultures exposed to large amounts of virus. Frozen virus-containing medium (-70°C) may be used for transducing cells. However, it should be thawed slowly on ice. If possible, we recommend to transduce cells with fresh virus-containing medium.
 8. The number of cells to seed varies considerably and depends on cell type, growth rate, passage, whether they are transformed or primary cells, and so forth. For each cell line, it is necessary to determine by preliminary assay the appropriate number of cells to seed.
 9. For transformed cells, hydroxyurea (20 mM) is added to reduce the scheduled DNA synthesis rate.

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The Use of Electrophoretic Mobility Shift Assays to Study DNA Repair

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1. Introduction

DNA repair pathways must include proteins that recognize and bind to damaged DNA. The search for such proteins has been facilitated by the use of electrophoretic mobility shift assays (EMSAs), which were first used to detect transcription factors that bind to specific DNA sequences (1,2). To study DNA repair, EMSAs have been adapted to detect proteins that bind to specific DNA lesions or structures.

In an EMSA, proteins bound to DNA can be resolved as distinctly migrating complexes by nondenaturing polyacrylamide gel electrophoresis. To analyze crude cellular extracts for proteins involved in DNA repair, a ^{32}P -labeled DNA probe is prepared so that it contains the DNA lesion or DNA structure of interest. To mask the effects of nonspecific DNA binding proteins, the DNA probe is incubated with cell extract in the presence of an excess of competitor DNA that does not contain the DNA structure.

For any EMSA, several general principles must be followed to ensure proper detection of the appropriate protein–DNA complexes. The procedure for extracting proteins from the cell nuclei must be optimized for the target protein: a cocktail of protease inhibitors must be present, particularly for high-molecular weight proteins that may be vulnerable to degradation; the salt concentration of the extraction buffer must be high enough to dissociate the target protein from DNA, but not so high that interfering components are also extracted. The electrophoresis conditions must be optimized to obtain a well-defined mobility shift: in particular, the salt concentration of the electrophoresis buffer can be adjusted to stabilize the specific protein–DNA complex while minimizing the formation of nonspecific complexes. Use of a minigel apparatus permits reso-

lution of the desired complexes in about 30 min; end-labeling of the probe DNA with ^{32}P permits autoradiographic detection of the complexes in a few hours. In our laboratory, gels are poured in lots of 13 and stored for up to 3 wk at 4°C so that experiments can be done with minimal setup time.

Once a complex is detected, its specificity for damaged DNA must be confirmed. If the mobility shifted complex contains a protein rather than some other biochemical molecule, complex formation should be sensitive to the addition of proteases. If the protein binds specifically to damaged DNA, a complex of the same mobility should not form with undamaged probe DNA. Alternatively, unlabeled competitor DNA with and without damage can be compared for their ability to compete away the labeled complex. The spectrum of DNA structures recognized by the protein can be determined by testing different competitor DNAs.

Of course, the discovery of a protein that binds to damaged DNA does not prove that it is involved in DNA repair. To establish biological significance, we have found it fruitful to screen candidate mutant cell lines for abnormalities in binding activity. Successful screening depends on procedures described below for rapidly making cell extracts from small numbers of cells.

This approach has been used to study a number of DNA repair proteins. In this chapter, we will describe our protocols for studying two such proteins (**Fig. 1**): xeroderma pigmentosum group E binding factor (XPE-BF), which is involved in the nucleotide excision repair of UV-induced damage, and Ku, which is involved in DNA double-strand break repair and V(D)J recombination.

In the case of XPE-BF, the probe DNA was a linear DNA fragment damaged by exposure to UV radiation, and the competitor DNA consisted of unlabeled linear DNA that was left undamaged (**3**). A complex with a well-defined mobility was detected even though UV radiation introduced DNA lesions throughout the length of the DNA probe. This occurred because the DNA probes employed were relatively short (<150 bp), and the mobility of the protein–DNA complex was determined primarily by the mobility of the protein in the nondenaturing gel (**4**). Specificity of XPE-BF binding activity was established by showing that the binding activity was competed away by the addition of unlabeled UV-damaged competitor DNA to the binding reaction (**Fig. 2A**). XPE-BF was then purified and partially sequenced in order to isolate a cDNA encoding a polypeptide of the expected molecular weight. To demonstrate that this polypeptide bound specifically to UV-damaged DNA, the cDNA was transcribed and translated *in vitro* so that it was labeled with ^{35}S -labeled methionine (**6**). A reverse EMSA was then used to show that the mobility of the labeled protein was specifically shifted by the addition of damaged DNA, but not by undamaged DNA (**Fig. 2B**).

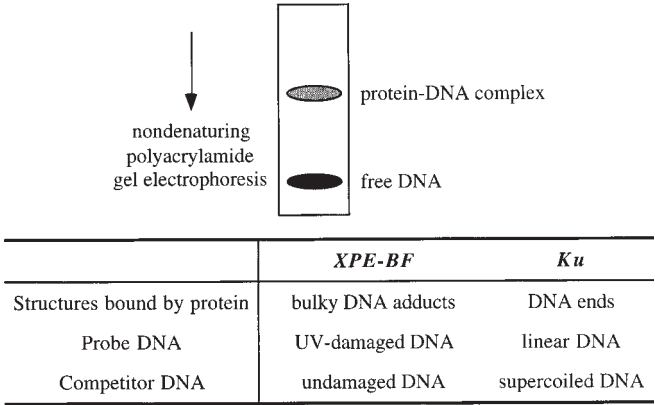


Fig. 1. EMSA for studying XPE-BF and Ku. UV-damaged DNA probe for XPE-BF or linear DNA probe for Ku may be incubated with cell extracts in the presence of the competitor DNA to block nonspecific binding. After incubation, the protein–DNA probe complex is resolved from the free DNA probe by nondenaturing gel electrophoresis.

To determine the biological relevance of XPE-BF, we screened a series of mutant cell lines known to be hypersensitive to UV radiation. A role in nucleotide excision repair was supported by the discovery that XPE-BF activity was absent in a subset of xeroderma pigmentosum group E cells, which are defective in this repair pathway (3). Furthermore, XPE-BF also recognized competitor DNA carrying intrastrand DNA crosslinks induced by the anticancer drug cisplatin, and levels of XPE-BF were found to be increased in extracts from cells selected for resistance to cisplatin (7). The biological relevance was established conclusively by showing that microinjection of XPE cells with purified XPE-BF restored DNA repair to the cells (8).

In the case of Ku, the probe was an undamaged linear DNA fragment, and the competitor DNA was supercoiled plasmid DNA (Fig. 3). This assay system detected a protein–DNA complex, which we originally denoted as DNA end-binding (DEB) factor . Binding specific for DNA ends was established by showing that the binding activity was competed away by the addition of unlabeled competitor plasmid DNA cleaved with any one of several different restriction enzymes, producing DNA ends with 5'-overhanging, 3'-overhanging, or blunt ends. The identification of DEB factor as Ku protein was first established by a useful adjunct to the EMSA, in which incubation of the binding reactions with anti-Ku antibodies produced a supershift of the original protein–DNA complex (10).

To determine the biological relevance of DEB factor, we screened a series of mutant cell lines hypersensitive to ionizing radiation, reasoning that a DNA

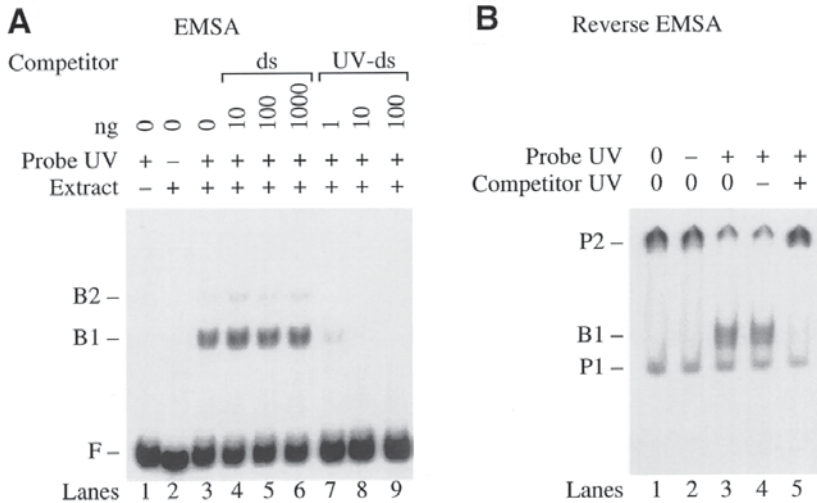


Fig. 2. EMSA and reverse EMSA for XPE-BF. **(A)** EMSA. Crude protein extracts from HeLa cells were incubated with f148 probe DNA in the presence of different amounts of unlabeled competitor DNA. The probe DNA was UV irradiated (lanes 1 and 3–9) or left intact (lane 2). Protein extract was omitted (lane 1) or added (lanes 2–9). Control binding reactions omitted competitor DNA (lanes 1–3). Competition was carried out with intact double-stranded DNA (ds) (lanes 4–6), and UV-irradiated double-stranded DNA (UV-ds) (lanes 7–9). F is free probe. B1 is protein-bound, shifted probe. The higher-order band B2 corresponds to two independent p125 binding events on the same probe (5). **(B)** Reverse EMSA. The fraction of ³⁵S-labeled p125 protein that bound to a UV-DNA cellulose column was assayed in a binding reaction with unlabeled f148 DNA. Probe DNA was omitted (lane 1), added as undamaged DNA (lane 2), or added as UV-damaged DNA (lanes 3–5). Unlabeled plasmid competitor DNA was either omitted (lanes 1–3), added as undamaged DNA (lane 4), or added as UV-damaged DNA (lane 5). P1 is ³⁵S-labeled p125. B1 is ³⁵S-labeled p125 bound to UV-irradiated probe DNA. Some of the ³⁵S-labeled p125 protein is retained in the well of the gel (P2) because it was purified from a UV-DNA cellulose column, so that some high molecular weight UV-damaged DNA coeluted as a complex with ³⁵S-labeled p125 protein.

end-binding protein might be involved in the repair of the double-strand breaks produced by ionizing radiation. This hypothesis was confirmed by the discovery that DEB factor was absent in three cell lines from X-ray complementation group 5, which is defective in double-strand break repair (10). Strikingly, these cells were also defective in V(D)J recombination, the pathway that generates immunological diversity by cleaving and rearranging the immunoglobulin genes in B-cells and the T-cell receptor genes in T-cells. Biological relevance

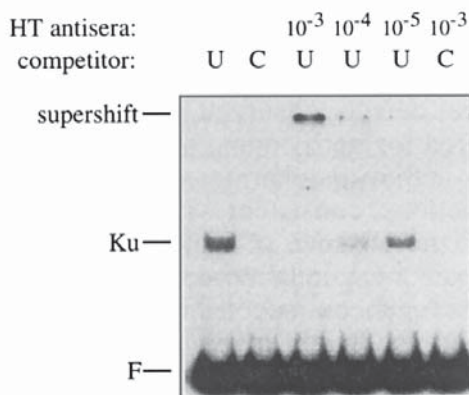


Fig. 3. EMSA and antibody supershift for Ku. Nuclear extract from the hamster cell line AA8 was incubated in the presence of radiolabeled f148 probe and uncut plasmid (U) DNA competitor in lane 1, or cut plasmid (C) in lane 2. The antiserum HT contains anti-Ku antibodies and was included in lanes 3–5 in dilutions ranging from 10^{-3} to 10^{-5} in the presence of uncut plasmid (U). The supershifted complex is specific for DNA ends as shown in lane 6, where cut plasmid (C) is the competitor.

was established by showing that transfection of the mutant cells with a cDNA expression vector for the 86-kDa subunit of Ku restored both ionizing radiation resistance and V(D)J recombination (*11,12*).

EMSAs have been used to identify other structure-specific proteins. The randomly damaged UV-irradiated DNA probe, which was used to detect XPE-BF in human extracts, failed to detect a corresponding factor in yeast extracts, but instead detected photolyase, which repairs UV-induced cyclobutane pyrimidine dimers by photoreactivation (*13*). Like XPE-BF, yeast photolyase recognized both UV-damaged and cisplatin crosslinked DNA. Although XPE-BF expression was associated with resistance to cisplatin, yeast photolyase conferred sensitivity to cisplatin (*14*). DNA probes with other structures have detected additional proteins. An oligonucleotide containing a single GT mismatch was used to identify the GT binding protein that has now been shown to be involved in mismatch repair (*15*). An oligonucleotide containing a single intrastrand cisplatin crosslink was used to search extracts for damage-specific DNA binding proteins that proved to be the HMG-1 and HMG-2 proteins (*16,17*). In this case, the proteins were not involved in DNA repair, but rather interfered with the nucleotide excision repair machinery and conferred sensitivity to cisplatin (*18*).

In conclusion, the EMSA has proven to be extremely useful for studying DNA repair. Our EMSA protocols for XPE-BF and Ku are described in detail,

because they remain useful for studying these proteins further, and because they are paradigms for how the EMSA might be used to discover new repair proteins or gain new insights into DNA repair in the future.

2. Materials

2.1. Probe DNA

2.1.1. Preparation of f148 probe

1. pRSVcat plasmid.
2. *Hind*III and *Pvu*II: 10 U/ μ L (New England Biolabs [NEB], Beverly, MA).
3. Agarose (IBI-Kodak, Rochester, NY).
4. 1X TBE: 89 mM Tris-HCl, pH 8.0, 89 mM borate, and 2 mM EDTA (*see Note 1*).
5. Ethidium bromide solution: 2 μ g/mL, dissolve in distilled water and filter through a 0.4- μ m filter.
6. Long-wavelength UV light source (366 nm): Model UVL-56 (Ultraviolet Products, Inc., San Gabriel, CA).
7. IBI-electroeluter: Model 46000 (IBI-Kodak).
8. Polyethylene tubing: id 1.6 mm.
9. Salt solution: 10 μ L of 0.5% bromophenol blue and 1 mL of 10 M ammonium acetate.
10. 18-Gage needle.
11. Razor blade.
12. Isobutanol.
13. Ethanol, 100, 80%
14. TE: 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA.

2.1.2. Labeling of f148 Probe: Klenow Method

1. 10X Klenow buffer: 100 mM Tris-HCl, pH 7.5, 50 mM MgCl₂, and 75 mM dithiothreitol (DTT).
2. 10 mM dATP, dGTP, and TTP.
3. α -³²P-dCTP: 3000 Ci/mmol, 10 mCi/mL (Amersham, Arlington Heights, IL).
4. DNA polymerase I, Klenow fragment: 5 U/ μ L (NEB).

2.1.3. Labeling of f148 Probe: Exonuclease III/Klenow Method

1. **Items 1–4 of Subheading 2.1.2.**
2. Exonuclease III: 100 U/ μ L, diluted in TE (NEB).

2.2. Cell Extracts

2.2.1. Whole-Cell Extract

1. Phosphate-buffered saline (PBS): 1 g/L D-glucose, 36 mg/L sodium pyruvate, 36 mg/L calcium phosphate, and 36 mg/L magnesium phosphate.
2. Lysis buffer: 700 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10 mM β -glycerophosphate, 2 mM MgCl₂, 10 mM KCl, 1 mM sodium vanadate, 1 mM phenylmethyl-

sulfonyl fluoride (PMSF), 1 mM DTT, 0.1% nonidet P-40 (NP-40), 10 µg/mL of each pepstatin, leupeptin, and aprotinin.

3. Protein assay kit (Bio-Rad, Hercules, CA).

2.2.2. Cytoplasmic/Nuclear Extracts

1. PBS (*see item 1, Subheading 2.2.1.*).
2. Buffer A without NP-40: 10 mM HEPES-KOH, pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, and 1 mM EDTA.
3. Buffer A with NP-40: 10 mM HEPES-KOH, pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, 1 mM EDTA, and 1.0 % (w/v) NP-40.
4. Buffer C: 20 mM HEPES-KOH, pH 7.4, 20% glycerol (v/v), 500 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 0.5 mM DTT.
5. 100X Protease inhibitor cocktail: 100 mM PMSF, 10 mg/mL pepstatin A, 10 mg/mL leupeptin, 10 mg/mL aprotinin.

2.3. Nondenaturing Gels

1. Minigel plates: 0.75 mm thickness, 80 × 100 mm² (Hoefer/Pharmacia, Piscataway, NJ).
2. Gel caster: SE215 Mighty small multiple gel caster (Hoefer/Pharmacia).
3. Acrylamide-*bis*-acrylamide: 29:1, (w/w) (Sigma, molecular biology-grade, St. Louis, MO).
4. 1X TGE buffer: 50 mM Tris-HCl, pH 8.5, 380 mM glycine, and 2 mM EDTA (*see Note 1*).
5. Ammonium persulfate: 10% of fresh solution.
6. TEMED.
7. Gel combs: 10- or 15-lane combs with 0.75 mm thickness (Hoefer/Pharmacia).

2.4. EMSA for Studying XPE-BF

2.4.1. UV-Damaged f148 DNA

1. Germicidal lamp: G15T8 (General Electric, Cleveland, OH).
2. Radiometer/photometer: Model IL1350 (International Light Inc., Newburyport, MA).

2.4.2. Competitor DNA to Mask Nonspecific Binding

1. Poly(dI-dC) (Pharmacia).
2. Salmon sperm DNA: sheared by passing through an 18-gage needle with pressure.
3. Linear plasmid DNA: pRSVcat plasmid digested with *Pvu*II and extracted with phenol/chloroform, and precipitated in ethanol.

2.4.3. Competitor DNAs to Test Specificity of Binding

1. Supercoiled pRSVcat plasmid.
2. AP treatment buffer: 0.01 M sodium citrate, 0.1 M NaCl, pH 5.0.
3. Pt treatment buffer: 3 mM NaCl, 1 mM NaH₂PO₄, pH 7.4.

4. *cis*-Dichlorodiammine platinum[II] (*cis*-DDP; cisplatin) (Sigma): Stock solution. Dissolve in Pt treatment buffer and store at -20°C in the dark.
5. Ethanol, 100%.
6. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Sigma): dissolve to 15 mM in ethanol and store at -20°C .
7. MNNG treatment buffer: 10 mM Tris-HCl, pH 7.5 and 50 mM NaCl.

2.4.4. Binding Assay

1. 5X binding buffer: 12 mM HEPES-KOH, pH 7.9, 60 mM KCl, 5 mM MgCl_2 , 4 mM Tris, 0.6 mM EDTA, 1 mM DTT, and 12% glycerol (v/v).
2. Bovine serum albumin (BSA): 10 mg/mL.
3. Plasmid DNA: pRSVcat, 1 mg/mL.
4. Poly(dI-dC)/salmon sperm DNA: 1.0 and 0.5 mg/mL, respectively.
5. Whole-cell or nuclear extract: 0.1–0.5 mg/mL.
6. Loading dye: 0.025% bromophenol blue in 5X binding buffer.
7. Whatman 3MM paper (Whatman, Clifton, NJ).
8. X-ray film: Kodak XAR-5 (Kodak).

2.5. Variation on EMSA: Reverse EMSA

2.5.1. In Vitro Synthesis of ^{35}S -Labeled p125

1. p125 plasmid: cloned into pCDNA3 vector (Invitrogen, Carlsbad, CA).
2. Rabbit reticulocyte lysate (Promega, Madison, WI).
3. ^{35}S -labeled methionine: 1000 Ci/mmol, 50 mCi/mL (Amersham).
4. T7 RNA polymerase buffer: 40 mM Tris-HCl, pH 7.9, 6 mM MgCl_2 , 2 mM spermidine, 10 mM DTT, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, and 0.5 mM UTP.
5. T7 RNA polymerase: 10 U/ μL (Promega).
6. Amino acid mixture minus methionine: 1 mM (Promega).
7. RNasin ribonuclease inhibitor: 40 U/ μL (Promega).
8. RNase-free water: treated with 0.1% DEPC at 37°C overnight and autoclaved at 120°C for 20 min.

2.5.2. Purification of Active ^{35}S -Labeled p125

1. UV-DNA cellulose resin: prepared as described previously (16).
2. Buffer D: 10 mM HEPES-KOH, pH 7.9, 2 mM EDTA, 2 mM DTT, 0.01% NP-40 (v/v).

2.5.3. Reverse Electrophoretic Mobility Shift Assay

1. Items 1–8, Subheading 2.4.4.
2. ^{35}S -labeled p125.
3. 50% Methanol and 10% acetic acid.
4. Amplify (Amersham).
5. Baby powder.

2.6. EMSA for Studying Ku

2.6.1. Probe

Materials for purifying and radiolabeling the f148 probe are described in **Subheading 2.1.**

2.6.2. Competitor DNA Against Nonspecific Binding

This consists of supercoiled pRSVcat plasmid: purified by CsCl centrifugation (**19**; *see* Chapter 8 for details on CsCl centrifugation).

2.6.3. Competitor DNAs to Test Specificity of Binding

1. Poly(dA) or poly(dT) (Pharmacia).
2. Double-stranded linear DNA: unlabeled f148 DNA.
3. Single-stranded circular DNA: phage M13 DNA (NEB).
4. Supercoiled pRSVcat plasmid: purified by CsCl centrifugation (**19**; *see* Chapter 8).

2.6.4. Binding Assay

1. **Items 1, 3, 6–8 of Subheading 2.4.4.**
2. Protein extract: 0.1–0.5 mg/mL.
3. Polyacrylamide gel: 4%.

2.7. Variation on EMSA: Antibody Supershift

1. Anti-Ku antibody: HT serum from a human autoimmune patient with polymyositis-scleroderma overlap syndrome (**10**).
2. 1% BSA.

3. Methods

3.1. Probe DNA

3.1.1. Preparation of f148 Probe

The 148 bp DNA fragment (f148) was isolated from a bacterial chloramphenicol acetyltransferase gene.

1. Digest pRSVcat plasmid with *Hind* III and *Pvu*II restriction enzymes.
2. Separate the digested DNA fragments on a 1.5% agarose gel.
3. Stain the gel with ethidium bromide solution and visualize the 148-bp DNA fragment (f148) with long-wavelength UV light. Long-wavelength UV is used instead of short-wavelength UV to prevent damage to the DNA.
4. Excise the band containing the f148 fragment with a razor blade.
5. Elute the f148 DNA fragment from the gel slice by using an IBI-electroeluter, as described below.
6. Soak the gel slice containing f148 in 0.2X TBE buffer for 5–10 min.
7. Preclear the electroeluter chamber by electrophoresis in 0.2X TBE for 20 min at 150 V. Carefully remove air bubbles in the chamber.

8. Place the gel slice in circular receptacle. Surround the gel slice with 0.2X TBE buffer, but do not cover the gel slice. Keep the valve up.
9. Flush the V-channel with 0.2X TBE buffer by using an 18-gage needle on a 1-mL syringe with polyethylene tubing to protect electroeluter device.
10. Underlay 125 μ L of salt solution into the V-channel.
11. Close the cover to move the valve to the intermediate position.
12. Run the electroeluter at 150 V; watch for the migration of the DNA band out of the gel slice with the handheld long-wavelength UV light.
13. Stop the electroelution when all of the DNA fragments have left the gel slice (approx 10–20 min).
14. Push the valve to the lowest position.
15. Withdraw the contents in the V channel and rinse the channel with 100 μ L of the salt solution.
16. Extract the eluate twice with 400 μ L of isobutanol, which will remove the contaminated dyes and reduce the volume.
17. Measure the final volume of the eluate, and add 1 vol of 100% ethanol and precipitate at -20°C for 2 h.
18. Spin the ethanol precipitate at 13,000g for 20 min at 4°C .
19. Wash the pellet with cold 80% ethanol solution.
20. Suspend the pellet in TE solution and measure the concentration (**19**).

3.1.2. Labeling of f148 Probe: Klenow Method (see **Note 2**)

1. Set up reaction mixture at room temperature as follows:

10X Klenow buffer	1 μ L
f148 DNA fragment (5 ng/ μ L)	4 μ L
10 mM dATP	0.5 μ L
10 mM dGTP	0.5 μ L
10 mM TTP	0.5 μ L
α - ^{32}P -dCTP (10 $\mu\text{Ci}/\mu\text{L}$)	1 μ L
Klenow (5 U/ μ L)	1 μ L
Distilled water to	10 μ L
2. Incubate at room temperature for 20 min.
3. Inactivate the Klenow enzyme by incubating the reaction mixture at 65°C for 10 min.
4. Purify the labeled f148 from unincorporated nucleotides by using spin-column chromatography (**19**).

3.1.3. Labeling of f148 Probe: Exonuclease III-Klenow Method (see **Note 3**)

1. Set up exonuclease III digestion reaction as below:

10X Klenow buffer	2 μ L
f148 DNA fragment (5 ng/ μ L)	4 μ L
Exonuclease III (0.2 U/ μ L, diluted in TE)	1 μ L
Distilled water to	10 μ L

2. Incubate at room temperature for 10 min.
3. Inactivate the exonuclease III by incubating the reaction mixture for 10 min at 65°C.
4. Add the following to the cooled reaction mixture at room temperature:

10 mM dATP	1 µL
10 mM dGTP	1 µL
10 mM TTP	1 µL
α - ³² P-dCTP (10 µCi/µL)	1 µL
Klenow (5 U/µL)	1 µL
Distilled water to	20 µL
5. Incubate at 37°C for 30 min.
6. Inactivate the Klenow by incubating the reaction mixture at 65°C for 10 min.
7. Purify the labeled f148 from unincorporated nucleotides by using spin-column chromatography (19).

3.2. Cell Extracts

3.2.1. Whole-Cell Extract

1. Harvest 2×10^6 cells from culture dishes in 1 mL of ice-cold PBS.
2. Pellet the cells by centrifugation for 1 min at 13,000g.
3. Resuspend the pellet in 30 µL of the lysis buffer.
4. Incubate the lysates at 4°C for 30 min with gentle shaking.
5. Centrifuge the lysates at 13,000g for 30 min at 4°C.
6. Save the supernatant at -80°C (see **Note 4**).
7. Measure the protein concentration by a modification of the Bradford method (20).

3.2.2. Cytoplasmic/Nuclear Extracts (see **Notes 5 and 6**)

1. Harvest 2×10^6 adherent cells from culture dishes in 1 mL of ice-cold PBS.
2. Wash the cells once with 500 µL of PBS.
3. Add 10 µL protease inhibitor cocktail to 1 mL of buffer A without NP-40. Wash the cells once with 500 µL of buffer A without NP-40.
4. Add 1 µL protease inhibitor cocktail to 100 µL buffer A with NP-40. Resuspend the pellets in 20 µL of buffer A with NP-40.
5. Incubate the suspensions at 4°C for 10 min with gentle shaking.
6. Spin down the lysates at 13,000g for 5 s at 4°C.
7. Save the supernatant as cytoplasmic extract at -80°C.
8. Wash the pellet in 300 µL of buffer A without NP-40 by gentle pipeting. Centrifuge at 13,000g for 5 s.
9. Add 10 µL of protease inhibitor cocktail to 1 mL of buffer C. Resuspend the pellet from **step 8** in 75 µL of buffer C.
10. Incubate the suspensions at 4°C for 20 min with gentle shaking.
11. Spin down the nuclear lysates at 13,000g for 15 min at 4°C.
12. Save the supernatant as nuclear extract at -80°C (see **Note 4**).
13. Measure the protein concentration by the method of Bradford (20).

3.3. Preparation of Nondenaturing Gel (see Note 7)

1. Assemble 13 sets of 0.75-mm thick minigels in a gel caster.
2. Prepare 4% polyacrylamide gel solution as follows:

Acrylamide- <i>bis</i> -acrylamide (29:1)	14 mL
5X TGE buffer	20 mL
Distilled water	65 mL
Ammonium persulfate (10%)	1 mL
TEMED	120 μ L
3. Gently mix the gel solution, and immediately pour to the top of the assembled gel caster with caution to avoid bubbles.
4. Tap the gel caster gently several times to remove the bubbles in the gel caster.
5. Insert the comb into the top of each gel.
6. Leave at room temperature for at least 4 h.
7. Disassemble the gel caster, wrap each gel with Saran Wrap, and store at 4°C until use. Be careful to remove residual polyacrylamide, which adheres to the outside of the individual gels—this can interfere with efficient electrophoresis (see Note 8).

3.4. EMSA for Studying XPE-BF

3.4.1. UV Irradiation of the f148 Probe (see Notes 9 and 10)

Damage the labeled f148 probe (0.2 μ g/mL) using a germicidal lamp at a flux of 10.4 J/m²/s for total doses of 100–5000 J/m².

3.4.2. Competitor DNA against Nonspecific Binding (see Note 11)

Crude cellular extracts have nonspecific DNA binding proteins including DNA end binding proteins. Thus, excess unlabeled linear double-stranded DNA is included in the reaction mixture. The amount of the competitor DNA is determined empirically because different extracts require different amounts of competitor DNA. A poly(dI-dC)/salmon-sperm DNA mixture (2:1, w/w) is generally used.

3.4.3. Competitor DNAs to Test Specificity of Binding (see Note 12)

The binding specificity for UV-damaged DNA and single-stranded DNA is measured by competition assay. The single-stranded competitor DNA is prepared by heating linear double-stranded DNA at 100°C for 5 min and then cooling rapidly in ice water. UV-damaged single-stranded DNA is prepared by UV-irradiating single-stranded DNA.

The chemically damaged competitor DNA is prepared as described previously (21). Apurinic DNA is prepared by incubating DNA (300 μ M DNA phosphate) in AP treatment buffer at 70°C for different times. Approximately one purine base is released every 4 min under these reaction conditions. Cisplatin-damaged

DNA is prepared by incubating supercoiled plasmid (300 μ M DNA phosphate) with *cis*-DDP at 37°C for 12–18 h in the dark. The DNA is purified by ethanol precipitation. MNNG is incubated with DNA (300 μ M DNA phosphate) for 24 h at 37°C in MNNG treatment buffer. The DNA is purified by ethanol precipitation.

3.4.4. Binding Assay

1. Assemble the reaction mixture as follows:

5X binding buffer	2 μ L
UV- ³² P-f148 probe	0.2 or 0.02 ng
BSA (10 mg/mL)	0.3 μ L
Plasmid DNA (1 mg/mL)	2 μ L
Poly(dI-dC)/salmon sperm DNA (1.0/0.5 mg/mL)	0.5–2 μ L
Distilled water to	8 μ L
Whole-cell or nuclear extract (0.1–0.5 mg/mL)	2 μ L

2. Add whole-cell or nuclear extract last.
3. Incubate the reaction mixture at room temperature for 30 min.
4. Add 2 μ L of loading dye to the reaction mixture, and gently mix together.
5. Resolve the protein–DNA complexes by nondenaturing gel electrophoresis at 10 V/cm at room temperature.
6. Dry the gel on Whatman 3MM paper, and expose to X-ray film at –80°C (**Fig. 2A**).

3.5. Variation on EMSA: Reverse EMSA

Specific binding of XPE-BF (p125) to UV-damaged DNA probe can also be visualized by a reverse mobility shift assay with labeled XPE-BF and unlabeled UV-f148 probe (**Fig. 2B**).

3.5.1. In Vitro Synthesis of ³⁵S-Labeled p125

Labeled p125 protein is synthesized by transcribing the p125 cDNA from a T7 promoter with T7 RNA polymerase and translating the mRNA in a rabbit reticulocyte lysate in the presence of (³⁵S) methionine.

1. Assemble the in vitro transcription/translation reaction mixture as follows:

pCDNA3 (p125) plasmid (1 mg/mL)	1 μ L
Rabbit reticulocyte lysate	25 μ L
(³⁵ S) methionine	4 μ L
T7 RNA polymerase buffer	2 μ L
T7 RNA polymerase	1 μ L
Amino acid mixture minus methionine	1 μ L
RNasin ribonuclease inhibitor	1 μ L
RNase-free water to	50 μ L

2. Incubate at 30°C for 120 min.
3. Store the in vitro translation products at –80°C.

3.5.2. Purification of Active ^{35}S -Labeled p125 Protein

All of the p125 protein synthesized in vitro is not active to bind UV-damaged DNA. Thus, the active fraction is purified through UV-DNA cellulose affinity chromatography.

1. Pack 10 μL of UV-DNA cellulose resin in a 200 μL Pipetman tip and equilibrate the column with buffer D.
2. Load the in vitro translated ^{35}S -labeled p125 proteins onto the column (50 μL of the reticulocyte lysate).
3. Wash the column extensively with 600 μL of buffer D containing 200 mM NaCl.
4. Elute the bound ^{35}S -labeled p125 protein with buffer D containing 1 M NaCl.
5. Store the fractions at -80°C .

3.5.3. Reverse EMSA

1. Assemble the reaction mixture as follows:

5X binding buffer	2 μL
UV-f148 probe	1 ng
BSA (10 mg/mL)	0.3 μL
Plasmid DNA (1 mg/mL)	2 μL
Poly (dI-dC)/salmon sperm DNA (1.0/0.5 mg/mL)	0.5 μL
Distilled water to	8 μL
^{35}S -labeled p125	2 μL

2. Incubate the reaction at room temperature for 30 min.
3. Add 2 μL of loading dye to the reaction mixture, and gently mix together.
4. Resolve the protein–DNA complex by nondenaturing gel electrophoresis at 10 V/cm at room temperature.
5. Fix the gel in 50% methanol and 10% acetic acid for 2 h.
6. Soak the gel in the Amplify for 30 min.
7. Dry the gel on Whatman 3MM paper.
8. Treat the sticky surface of the dried gel with baby powder, which allows direct contact of the gel with the X-ray film.
9. Expose the gel to X-ray film at -80°C .

3.6. EMSA for studying Ku

3.6.1. Probe

Prepare the f148 probe as described in **Subheading 3.1.1.** and radiolabel the probe as described in **Subheading 3.1.2.** (see **Notes 13** and **14**).

3.6.2. Competitor DNA Against Nonspecific Binding (see **Note 15**)

Crude nuclear extracts have proteins that are not specific for DNA ends that may bind to the labeled f148 probe. To minimize the effects of these proteins on the EMSA, supercoiled plasmid is included in the binding reaction. Because the supercoiled pRSVcat plasmid contains the f148 fragment, but has no free

ends, it is a good choice for being a nonspecific competitor. In general, human cell extracts require 2 μg of nonspecific competitor in a 10- μL reaction. Rodent cell extracts, however, require only 50–100 ng of nonspecific competitor.

3.6.3. Competitor DNAs to Test Specificity of Binding

Perform competition assays using single-stranded linear DNAs (poly[dA] or poly[dT]), double-stranded linear DNA (unlabeled f148), single-stranded circular DNA (phage M13), or supercoiled double-stranded DNA (pRSVcat). These competitor DNAs should be included in the binding reaction in concentrations ranging from 0.2–200 ng. Competitors containing free DNA ends (unlabeled f148) or DNA containing single-stranded to double-stranded DNA transitions (phage M13) compete for Ku binding activity.

3.6.4. Binding Assay

1. Prepare the reaction mixture as follows:

5X binding buffer	2 μL
f148 probe	0.2 ng
Supercoiled plasmid	2 μg (for human extracts) or 50–100 ng (for rodent extracts)
Distilled water to	8 μL
Nuclear extract	0.5 μg

2. Incubate the reaction for 5 min at room temperature.
3. Add 2 μL of loading dye to the reaction, and mix by gentle pipeting.
4. Resolve the protein–DNA complexes by nondenaturing gel electrophoresis at 10 V/cm at room temperature.
5. Dry the gel on 3MM Whatman paper and expose to X-ray film at -80°C .

3.7. Variation on EMSA: Antibody Supershift

Anti-Ku antibodies may be added to the binding reaction to supershift the Ku/DNA complex. Serial dilutions of the antibodies are made in 1% BSA and added to the reaction mix above, prior to adding the protein extract (**Fig. 3**).

4. Notes

1. The high-salt buffers TGE and TBE are superior to low-salt buffers in these EMSAs, because the high-salt buffers reduce nonspecific binding more efficiently.
2. When labeling the DNA probe with Klenow fragment, the radiolabeled nucleotide used to fill in the 5'-overhang of the DNA should be complementary to an internal base of the 5'-overhang. Inefficient labeling may occur if the radiolabeled nucleotide is complementary to the last base of the 5'-overhang because of exonuclease activity in the Klenow fragment.
3. The specific activity of the probe can be increased by using the exonuclease III/Klenow method. This can be helpful in detecting XPE-BF in rodent cells, where levels are substantially lower than in human cells.

4. Extracts may be stored at 4°C for 1–3 d, but for longer periods, they should be stored at –80°C. Extracts should be stored in aliquots to minimize repeated freeze–thawing. Thawing of the extracts should be done on ice to minimize protease activity, which may occur at higher temperatures.
5. The extraction procedure can be modified by using different salt concentrations in buffer C or changing the NP-40 concentration in buffer A.
6. Carryover of NP-40 to the extraction buffer (buffer C) can inhibit extraction of larger proteins. Washing the nuclei following the treatment with buffer A containing NP-40 removes the NP-40 and overcomes this effect (**Subheading 3.2.2., step 8**). Additionally, larger proteins may be affected by proteases to a greater extent than smaller proteins, making it critical that protease inhibitors be present at all steps in the extraction procedure following the lysis in buffer A.
7. Lowering the acrylamide concentration and decreasing the length of the DNA probe can facilitate the detection of larger DNA/protein complexes by permitting the migration of these complexes into the gel.
8. Conduction leaks in the gel can lead to aberrant migration of the free probe and protein/DNA complexes. This can be minimized by cleaning areas around the gel and gel box where salt buildup can occur.
9. In detecting XPE-BF, increasing the UV dose on the probe DNA can be utilized to increase the number of lesions per DNA molecule. Higher-order complexes corresponding to multiple XPE-BF binding events can be visualized at these higher doses (5).
10. When cyclobutane pyrimidine dimers were removed from a UV-damaged DNA probe by treatment with purified photolyase, the binding was decreased compared to the untreated UV-damaged DNA probe. This suggests that XPE-BF recognizes at least some cyclobutane dimers (5).
11. Because these EMSAs rely on the structure-specific binding properties of the DNA repair proteins, it is important that the competitor DNAs be free of contaminating structures that might compete for binding activity.
12. XPE-BF recognizes DNA damaged by several agents, including UV irradiation, but direct binding was observed only to the UV-damaged double-stranded DNA probe. The binding of XPE-BF to apurinic sites, cisplatin adducts, nitrogen mustard adducts, and single-stranded DNA was detected by showing that these forms of damaged DNA could compete with UV-damaged DNA for binding to XPE-BF (5,21). XPE-BF may have low affinities for these forms of DNA, so that binding activity could only be detected in the competition assay, but not the direct binding assay.
13. Increasing the length of the radiolabeled DNA probe can be utilized to detect multiple Ku binding events. This is possible because Ku is able to bind DNA and translocate along the molecule. Thus, longer DNA lengths permit more Ku molecules to bind and produce a “ladder” pattern in the EMSA (9).
14. Decreasing the length of the DNA probe can allow a decrease in the amount of competitor DNA used in the EMSA for Ku.
15. The competitor used in the EMSA for Ku should be supercoiled DNA, which is free of contaminating nicked or linear DNA. Ku binds strongly to free DNA ends

and has been reported to bind nicks, so competitors with these structures will obscure Ku binding activity.

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Byung Joon Hwang and Vaughn Smider contributed equally to this chapter.

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Mismatch Repair Assay

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1. Introduction

DNA mismatch repair plays an important role in mutation avoidance by recognizing and correcting mismatched bases and loops prior to their fixation as mutations. When mismatch repair is defective, cells exhibit elevated rates of spontaneous mutations. For example, microsatellite sequences exhibit frequent gains or losses of simple repeat units in tumor cells from patients with hereditary nonpolyposis colon cancer (HNPCC; **1–3**). Defects in mismatch repair have also been identified as an important route to tolerance of certain cytotoxic methylating agents (reviewed in **4**). Cell lines that are deficient for methyltransferase activity normally exhibit extreme sensitivity to *N*-methyl-*N*-nitrosourea (MNU) or 1-methyl-3-nitro-1-nitrosoguanidine (MNNG). However resistant subpopulations can arise spontaneously, and these subpopulations have been shown to be mismatch repair defective (**5,6**). To determine unambiguously that both HNPCC (**7**) and alkylation tolerance were owing to mismatch repair-defects, it was crucial to prove that mismatch repair activity was reduced or eliminated in mutant cells.

The identification of mismatch repair deficiencies has been greatly facilitated by a specific biochemical assay (**8,9**) for mismatch correction activity in cellular extracts (**Fig. 1**). The specificity of the assay stems from the design and production of pure, well-defined mismatched substrates (**8,9**). These DNA molecules harbor two crucial elements: the mismatch and a strand break (**Fig. 1A**). The mismatch can be any single-base or looped mispair, but typically a G-T lesion is utilized, because it is efficiently repaired in all systems. The presence of the mismatch stimulates protein binding and excision reactions that are essential for repair. The strand break is necessary for reactivity in eukaryotic extracts, presumably by acting as an entry point for helicases and/or exonu-

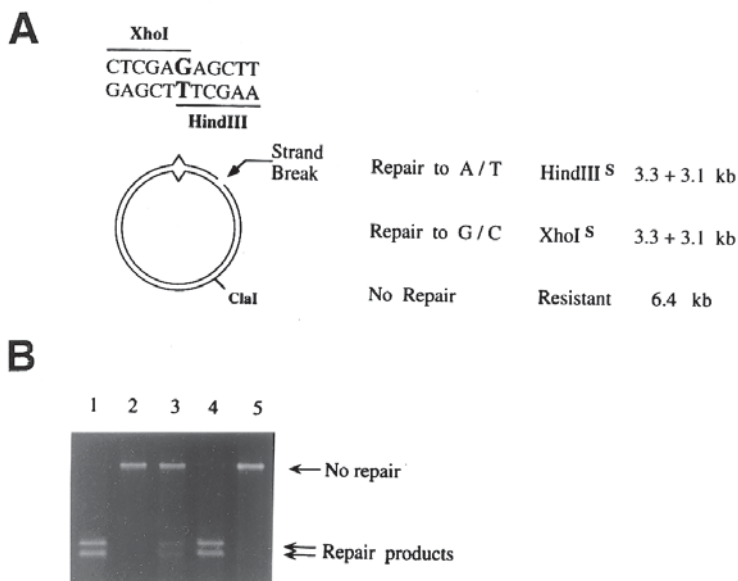


Fig. 1. Heteroduplex DNA substrate and its correction by mammalian nuclear extracts. Panel **A** is a schematic diagram of the 6.4-kb heteroduplex substrate. The sequence of the mismatched base pair (triangles) is expanded to indicate the location of restriction sites (underlined). The position of the strand break corresponds to an *Sau96I* site 125 bp 5' to the mispair. Expected restriction fragment sizes of repaired and unrepaired DNA are shown. Panel **B** is an example of a mismatch repair assay. DNA (0.1 μ g) and mammalian cell extract (SO cells, 50 μ g) were incubated, and the DNA isolated and treated with restriction enzymes (lanes 1–3 cut with *HindIII* + *ClaI*; lanes 4–5 cut with *XhoI* + *ClaI*). Lane 1, MR1 RF; lane 2, negative control for G-T; lane 3 repair of G-T; lane 4, MR3 RF; lane 5, lack of repair of G-T.

cleaves. The net result of the strand break is to direct repair to the discontinuous strand (9,10).

The assay for mismatch repair activity is based on restoration of restriction enzyme cleavage sites to the heteroduplex substrate (Fig. 1A). The G-T mispair resides within overlapping sites for *HindIII* and *XhoI* (8). The presence of the mispair confers resistance to both enzymes, so the unrepaired heteroduplex is not cut. If the mismatch undergoes correction upon incubation with cellular proteins, the DNA becomes sensitive to one or the other restriction endonuclease. Display of the cleaved DNA on an agarose gel allows quantitation of both the extent of correction as well as the strandedness of the reaction. In assays of eukaryotic extracts, the strand break in the heteroduplex substrate targets repair to a specific product. In the example shown in Fig. 1A, the break directs mismatch-stimulated excision to the G-containing, outer strand, result-

ing in gap formation. DNA repair synthesis utilizing the inner strand as template will regenerate the *Hind*III site. Mismatch correction is expected to yield significant amounts of *Hind*III-sensitive product, with little or no *Xho*I-sensitive DNA.

One key to successful assays for mismatch repair is production of high-quality DNA heteroduplex (**Fig. 2A**). This DNA can be prepared in multimicrogram amounts and stored for many months at 4°C (**8**). The assay is performed with 0.1 µg of mismatched substrate, so numerous experiments can be performed with one preparation. The substrate is constructed by annealing DNA strands of bacteriophage f1 (a close relative of M13) that differ by 1 bp (**Fig. 2A**). The G-containing strand comes from the double-stranded replicative form (RF) phage MR3. The RF DNA is converted from circular to linear form by cleavage with the commercially available restriction enzyme *Sau*96I. The T-containing strand arises from the single-stranded viral DNA of a point mutant variant, MR1. (Both types of DNA can be readily isolated in large amounts, and protocols for these isolations are included in **Subheading 3**. The original phage was first described by Modrich and colleagues (**8**), and these authors should be contacted for small aliquots of phage MR1 and MR3.) On strand annealing, the mismatched G-T substrate is formed. The requisite strand break corresponds to the *Sau*96I site of linearization of the RF DNA. Therefore, this molecule contains the two key components for mismatch repair assays. Some purification is required to remove the excess single-strand DNA (ssDNA) and the small amount of linear duplex that forms on reannealing (**Fig. 2A,B**). Protocols for purification are included below.

Extracts from the cells of interest provide the source of mismatch repair proteins. Fortunately, nuclear extracts can be prepared by well-established techniques (**9**), so we shall not dwell on this point. Given adequate amounts of high-quality substrate and nuclear extract, the assay for mismatch correction activity is straightforward. Heteroduplex DNA is incubated with cell extract (50–100 µg protein in a 10-µL volume) in the presence of a mixture of cofactors (ATP, Mg²⁺, and others) at 37°C for 15 min. The DNA is recovered by proteinase K treatment, phenol and chloroform extractions, and ethanol precipitation. Repair is scored by restriction cleavage with *Hind*III plus *Cla*I (for repair of the nicked strand). The purpose of the *Cla*I is to linearize all DNA molecules. If the DNA has undergone repair, the 6.4-kb linear *Cla*I fragment will be cleaved by *Hind*III to 3.3 + 3.1 kb pieces (**Fig. 1A**). If desired, correction on the continuous strand can be determined by cleavage with *Xho*I plus *Cla*I. The *Xho*I control provides evidence that repair is nick-directed, and therefore owing to *bona fide* mismatch correction, but this experiment is optional. Analysis of the cleavage products on a standard agarose gel (**Fig. 1B**) allows ready visualization of the result. The extent of repair is determined by measur-

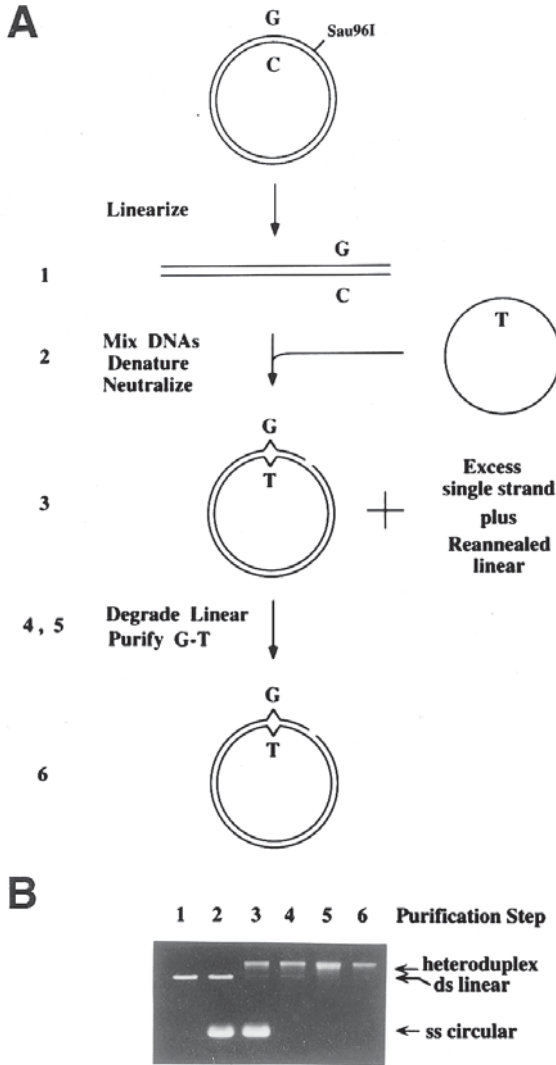


Fig. 2. Preparation of heteroduplex DNA. Panel **A** illustrates schematically the procedure for construction of the mismatched substrate. Panel **B** is an agarose gel of the various stages of the preparation. Aliquots containing approx 0.1 μ g dsDNA were loaded onto a 1% agarose gel buffered in TBE and run at 60–70 V for approx 3 h. The numbers at the top of each lane correspond to the DNA species denoted in panel **A**.

ing the intensities of the 3.3 + 3.1 kb repair bands as a percentage of the total intensity (6.4 + 3.3 + 3.1 kb bands). Quantitation of repair can be achieved using direct fluorescence readers or by photographing the gel and scanning the

photographic negative on a densitometer. To convert to moles of repair product, 100% correction of 0.1 μ g corresponds to 24 fmol of product.

An example of a mismatch repair assay is shown in **Fig. 1B**. Lanes 1 and 4 are control reactions in which nonmismatched RF DNAs were incubated with extract and the DNA was isolated and treated with restriction enzymes. Complete cutting to 3.3 + 3.1 kb products by *Hind*III + *Cla*I (lane 1) or *Xho*I + *Cla*I (lane 4) proves that restriction of RF DNA proceeds to completion under these conditions. Therefore, repair products arising from the heteroduplex will also undergo cleavage. Lane 2 is an important negative control. The G-T heteroduplex and cell extract were incubated separately, then mixed, and immediately quenched by addition of proteinase K and SDS. Under these conditions, no mismatch repair can occur. When challenged with *Cla*I + *Hind*III, only the 6.4-kb linear band owing to *Cla*I cleavage is observed. This control proves that cleavage by *Hind*III is precluded by the presence of the mismatch. Since many restriction enzymes can, at high concentrations, partially cleave mispaired sites, it is important to elucidate the baseline level of cutting in these control reactions. Lanes 3 and 5 display the products observed when heteroduplex and extract are incubated together and subsequently restricted. Lane 3 shows approx 30% repair of the heteroduplex, as judged by intensity of the *Hind*III-sensitive form as a percentage of the total DNA in the lane. Note the clear difference of product formation in lane 3 compared to the negative control in lane 2. In contrast, repair on the continuous strand is limited (<5%), as shown by the minimal amount of DNA that becomes sensitive to *Xho*I (lane 5).

2. Materials

2.1. General Reagents

1. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
2. 10- and 23-mm Dialysis tubing, mol-wt cutoff 6000–8000 (Spectra/Por).
3. Buffered phenol (pH 6.8–7.0), stored at 4°C for no more than 2 wk.
4. Chloroform/isoamyl alcohol mixture (24:1).
5. Cesium chloride, molecular biology-grade (Sigma, St. Louis, MO).
6. 10 mg/mL Ethidium bromide stock in water, stored at 4°C.
7. 18-Gage hypodermic needles.
8. 1X TBE electrophoresis buffer: 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0.
9. 2X TY media: 1% yeast extract, 1.6% Bacto-tryptone, 0.5% sodium chloride.

2.2. Viral DNA Isolation

1. Titered stock of desired f1 bacteriophage.
2. Fresh culture of JM101 cells.
3. Polyethylene glycol, mol-wt 8000 (Sigma).
4. Sodium chloride (Sigma).

5. STE buffer: 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0.
6. TEN buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl.

2.3. RF DNA Isolation

1. Titered stock of desired f1 bacteriophage.
2. Fresh culture of JM101 cells.

2.4. Heteroduplex Annealing Reaction

1. 1 mg Viral DNA.
2. 200 µg RF DNA, linearized with *Sau96I* (New England Biolabs; NEB, Beverly, MA).
3. 4 M Sodium chloride.
4. 1 M Tris-HCl, pH 7.6.
5. 10 N Sodium hydroxide.
6. 2.9 N Glacial acetic acid (sixfold dilution of glacial stock).
7. 1 M Potassium chloride.
8. 1 M Potassium phosphate, pH 7.5.

2.5. Hydroxylapatite Column Purification

1. Hydroxylapatite column resin (BioGel HTP Gel, Bio-Rad, Hercules, CA).
2. Potassium phosphate buffers at pH 6.9: 30, 140, and 420 mM.

2.6. DNase Digestion

1. ATP-dependent DNase (ExoV, Amersham Life Sciences, Arlington Heights, IL).
2. 10X ExoV buffer: 667 mM glycine-NaOH, pH 9.4, 50 mM MgCl₂, 83 mM β-mercaptoethanol.
3. 100 mM ATP stock, neutralized to pH ~7.0 with 1 N NaOH.

2.7. Repair Reaction

1. 10X Repair buffer: 200 mM Tris-HCl, pH 7.6, 500 µg/mL acetylated bovine serum albumin (BSA), 25 mM ATP, 10 mM glutathione (reduced), 1 mM each dNTP (neutralized), 100 mM MgCl₂.
2. 1 M HEPES, pH 7.5.
3. 1 M KCl.
4. 0.1 µg DNA heteroduplex/reaction.
5. 50–100 µg Cell extract/reaction.

2.8. DNA Purification

1. Stop buffer: 88 µg/mL proteinase K, 24 mM EDTA, pH 8.0, 0.7% sodium dodecyl sulfate (SDS).
2. Ether.
3. 1 mg/mL tRNA.
4. 3 M Sodium acetate, pH 7.0.
5. Ethanol, 70%, 100%, stored at -20°C.

2.9. Restriction Endonuclease Analysis

1. 1 mg/mL RNaseA (DNase-free).
2. Restriction enzymes: *Cla*I, *Hind*III, *Xho*I (NEB).
3. 10X Restriction buffer #2 (NEB).
4. 100X Acetylated BSA (NEB).
5. Gel-loading buffer: 50% glycerol, 50 mM EDTA, 1% SDS, 0.08% xylene cyanol, 0.08% bromophenol blue.
6. 1% Agarose gel in 1X TBE.

3. Methods

3.1. Viral DNA Isolation

1. Both viral and RF DNA sufficient for one to two heteroduplex preparations can be obtained from 4 L of phage-infected bacteria (*see Note 1*). Preparation of a G-T heteroduplex requires ssDNA from f1 bacteriophage MR1 and RF DNA from f1 bacteriophage MR3. Grow a 5-mL overnight culture of JM101 cells at 37°C. Dilute this culture into fresh 2X TY media to an OD₆₀₀ of 0.1. Prepare 1 mL of diluted culture for every 1 L to be grown (4 mL total). Grow the diluted cells to an OD₆₀₀ of 0.3–0.4. This density of cells is equivalent to approx 0.5×10^8 cells/mL. Add enough f1 phage stock to the cells to give a 5:1 ratio of phage to bacterial cells, respectively. Therefore, 4 mL of cells at the given density will require 1.0×10^9 plaque forming units (*pfu*) for optimal infection. Titers of phage stocks are generally 10^{12} *pfu*/mL; a dilution of this stock may be necessary to give a reliable pipeting volume. Allow the phage to infect the cells with incubation of the mixture at 37°C for 10 min without shaking. After the incubation, add 1 mL of the infected JM101 cells to 1 L of 2X TY media. Grow with shaking overnight (16 h).
2. To pellet the JM101 cells, centrifuge the cultures in a GSA rotor at 10,000 rpm (16,300g) for 15 min at 4°C. The pelleted cells contain the RF DNA, and can be stored in STE buffer at –20°C (*see Subheading 3.2.*). The clear supernatant should be poured off carefully into a clean Erlenmeyer flask that is large enough to allow the addition of 160 g of PEG 8000 and 120 g of NaCl/4 L of supernatant. Stir the supernatant for 60 min at room temperature to allow the PEG and NaCl to go into solution. The supernatant should become turbid as the phage particles precipitate. For the best precipitation, stir the solution overnight at 4°C.
3. To pellet the phage particles, centrifuge the supernatant solution at 4000 rpm (2600g) in a GSA rotor for 15 min at 4°C. Drain away the supernatant, and resuspend the pellet in 4 mL of TEN buffer/1 L of supernatant. Add 4.04 g of cesium chloride/10 mL of phage solution. Place this mix into a sealable ultracentrifuge tube, and centrifuge in a Ti70.1 rotor at 54,000 rpm (287,000g) for 20 h at 4°C. The finished gradient will contain an opalescent band approximately halfway down the tube; this is the viral DNA. Retrieve this band from the tube using a syringe and an 18-gage needle to prevent DNA shearing. Be careful to avoid the brown protein band that forms below the DNA band in the gradient. Dialyze the DNA against TE buffer (*see Note 2*).

4. Extract the dialyzed DNA sequentially with equal volumes of buffered phenol, phenol/ CHCl_3 -isoamyl alcohol, and then CHCl_3 -isoamyl alcohol. Add 1/10 vol of 3 M NaOAc, pH 7.0, and 2 vol of 100% EtOH. Store at -20°C for 1–2 h, then pellet the DNA in an SS-34 rotor at 10,000 rpm (12,000g) for 15 min at 4°C . Air dry the pellet and resuspend in approx 3 mL of TE. Determine the concentration of the DNA at A_{260} . An A_{260}/A_{280} ratio should be between 1.8 and 2.0 (see **Note 3**). An average yield of DNA from a 4-L culture is 3 mg.

3.2. RF DNA Isolation

The infection of JM101 cells and culture growth is performed as described in **step 1** of **Subheading 3.1**. To pellet the cells containing the RF DNA, centrifuge the cultures in a GSA rotor at 10,000 rpm (16,300g) for 15 min at 4°C . Resuspend and wash the cells in 100 mL STE buffer/1 L culture. Pellet the cells at 4000 rpm (2600g) for 15 min at 4°C . The RF DNA can now be isolated from the cells using a standard alkali-lysis procedure and subsequent purification on a CsCl gradient (**11**) (see **Note 4**). This DNA isolation method was chosen because the purity of the DNA is crucial for the heteroduplex preparation. An average yield of DNA from a 4-L culture is 1 mg.

3.3. Heteroduplex Preparation and Purification

The heteroduplex DNA substrate is formed by annealing viral DNA and linearized RF DNA that differ in sequence at only one base (**Fig. 2A**). To have efficient heteroduplex formation, the viral and RF DNA molecules are mixed at a 5:1 (w/w) ratio, respectively. Subsequent steps remove the excess ssDNA and any remaining reannealed linear RF, leaving only the desired heteroduplex substrate.

1. Linearize 200 μg RF DNA with the restriction endonuclease *Sau96I*. Use of this enzyme will place the nick 125 bases 5' to the mispair, an optimal distance for efficient repair (**7**). Extract the restricted DNA with an equal volume of buffered phenol, phenol/ CHCl_3 -isoamyl alcohol and CHCl_3 -isoamyl alcohol. Then ethanol precipitate the DNA. If the proteins from the restriction reaction are not removed, the efficiency of the annealing reaction will decrease significantly.
2. Annealing: At room temperature, combine 1 mg of ssDNA with 200 μg of linearized RF DNA. To this add 10 μL of 4 M NaCl and 200 μL of 1 M Tris-Cl, pH 7.6. Bring the total volume to 4 mL with sterile double-distilled water (ddH_2O). Add to this mixture 120 μL of 10 N NaOH, made fresh, to denature the DNA. Mix gently, and then allow the solution to stand at room temperature for 5 min. Neutralize the solution by adding 400 μL of 2.9 M acetic acid, 540 μL of 1 M KCl, and 496 μL of 1 M potassium phosphate (KP_1), pH 7.5. Mix gently, then incubate the solution at 65°C for 30 min and then 37°C for 60 min. Place the annealed mixture on ice. To determine the efficiency of annealing, analyze the reaction on a 1% agarose minigel (see **Note 5**). In general, about 90% of the starting ds linear DNA should be converted to heteroduplex substrate (**Fig. 2B**, compare lanes 2 and 3).

3. In preparation for the first purification step, the annealing reaction mix must be dialyzed against 30 mM KPi , pH 6.9 (*see Note 6*). Place the reaction (~6 mL) in 23-mm dialysis membrane and dialyze at 4°C. The efficiency of DNA binding to the hydroxylapatite column will be greatly improved by having the sample equilibrated in this buffer.
4. The use of sequential hydroxylapatite (HAP) columns allows for the separation of most of the excess ssDNA from the heteroduplex substrate (*see Note 7*). Pour two 3-mL HAP columns, which are equilibrated in 30 mM KPi , pH 6.9. These columns work best when the HAP resin is “de-fined” six or seven times before being poured in the column. Load the annealed reaction mix (~6 mL), which is now in 30 mM KPi buffer. Collect the flowthrough, and reload it onto the column; this increases the efficiency of DNA binding. Wash the column with one column volume of 30 mM KPi buffer. Load one column volume of 140 mM KPi , pH 6.9; this concentration of buffer will elute the ssDNA. Wash the column with three column-volumes of 30 mM KPi buffer. Load two column volumes of 420 mM KPi , pH 6.9, buffer. This will elute the heteroduplex substrate, linear double-stranded DNA (dsDNA) and any remaining ssDNA. To confirm the elution of each DNA species, an aliquot from each fraction can be analyzed on a 1% agarose gel. The heteroduplex will still be contaminated with a substantial amount of ssDNA. Combine the fractions containing the heteroduplex substrate, and dialyze them overnight against 30 mM KPi , pH 6.9. This will prepare the sample for loading on the second HAP column, which will remove almost all the remaining ssDNA (**Fig. 2B**, Lane 4). The procedure for running the second HAP column is the same as outlined above. Combine the fractions containing heteroduplex substrate, and dialyze against TE buffer (*see Note 8*). After the phosphate buffer is removed, add 1/10 vol of 3 M NaOAc, pH 7.0, and 2 vol of 100% EtOH. Precipitate the DNA at -20°C overnight.
5. The ATP-dependent DNase, ExonucleaseV, is an enzyme that will specifically degrade any residual linear dsDNA found in the heteroduplex preparation. Pellet the ethanol-precipitated heteroduplex DNA by centrifugation at 10,000 rpm (13,300g) in an SS-34 rotor for 15 min at 4°C. Let the DNA pellet air-dry, and then resuspend it in 2 mL sterile ddH₂O. Add 255 μL of 10X ExoV buffer and 10.8 μL of 100 mM ATP (450 μM final concentration). Prewarm this solution at 37°C for 5 min. Add 24 U of ExoV enzyme and incubate the solution for 90 min at 37°C. Place the solution on ice, and analyze an aliquot of the reaction on a 1% agarose minigel (**Fig. 2B**, compare lanes 4 and 5; *also see Note 9*). Once degradation of the linear dsDNA is complete, add 45 μL of 0.5 M EDTA to quench the reaction.
6. Cesium chloride (CsCl) purification: After the DNase treatment of the heteroduplex preparation, it is necessary to remove any residual ssDNA plus small molwt products from the ExoV reaction. Use of a CsCl gradient will complete the purification as well as concentrate the substrate into a smaller volume. To prepare the gradient, bring the heteroduplex preparation to 4 mL total with TE buffer. Add 4.2 g of CsCl and 400 μL of 10 mg/mL ethidium bromide. Load the sample

into a sealable ultracentrifuge tube, and centrifuge at 45,000 rpm (194,000g) for 16 h at 20°C in a Ti65V rotor. A handheld long-wave (365 nm) UV light can be used to visualize the DNA band in the gradient, but exposure should be kept to a minimum as the combination of UV-light and ethidium bromide will promote DNA damage. There should be only one visible band, located approx 1/3–1/2 of the way down the tube. Retrieve the band using an 18-gage needle to prevent DNA shearing. To remove the ethidium bromide, extract the sample four to five times with equal volumes of water-saturated butanol. Then dialyze the sample against 1 L of TE buffer to remove the CsCl. The initial dialysis should be performed at room temperature to prevent precipitation of the CsCl; subsequent dialysis is done at 4°C. Analysis of the purity of the heteroduplex DNA can be monitored on a 1% agarose gel (**Fig. 2B**, lane 6). If all the ssDNA is not removed at this point, a second CsCl gradient is recommended (*see Note 10*). With the recommended amounts of starting viral and RF DNA, it is possible to obtain 20–40 µg of pure heteroduplex substrate from this procedure (*see Note 11*).

3.4. *In Vitro* Repair Assay

1. This assay combines the G-T heteroduplex substrate with human cell extracts to examine repair of the mismatch. Each reaction has a total volume of 10 µL and should contain: 0.1 µg heteroduplex substrate (24 fmol), 50–100 µg cell extract, 1X repair buffer, 20 mM HEPES-KOH, pH 7.5, and 100 mM KCl. The samples should remain on ice during the addition of the reagents. The complete reaction mix is incubated for 15 min at 37°C. To stop the repair reaction, add 40 µL of stop buffer, and incubate this mix at 37°C for 15 min. An important control is to incubate the DNA separately from the remaining components (including extract), then mix the samples, and add stop buffer immediately. This negative control allows assessment of the resistance of the heteroduplex to restriction digestion (**Fig. 1B**, lane 2).
2. The repaired heteroduplex substrate must be purified from the cell extracts and reaction buffers before the DNA can be analyzed with restriction endonucleases. After incubation with stop buffer, add 50 µL TE buffer. Extract the samples once with an equal volume (100 µL) of buffer-saturated phenol, once with 100 µL of CHCl₃-isoamyl alcohol, and then twice with 400 µL of ether (*see Note 12*). Place the samples at 37°C for 15 min to evaporate the residual ether. Precipitate the DNA with 1 µL of 1 mg/mL tRNA, 10 µL of 3 M NaOAc pH 7.0, and 300 µL of 100% ethanol at –20°C for 30 min. Pellet the DNA in a microcentrifuge for 15 min at 4°C. Remove excess salts with a cold 70% ethanol wash. Dry the DNA pellets under vacuum for 10 min.
3. Restriction analysis: Resuspend the DNA pellets with vortexing in 14 µL of a restriction buffer mix composed of: 15 µL of 10X restriction buffer #2, 10 µL of 1 mg/mL RNase A, 1.5 µL 100X of BSA-acetylated and 113.5 µL of ddH₂O (enough for 10 samples). The restriction enzymes should be added individually; 2.5 U of *Cla*I to all samples plus 2 U of *Hind*III or *Xho*I, depending on the strand of the substrate being analyzed (*see Note 13*). Digest the samples for 60 min at

37°C. Add 2 μ L of gel-loading buffer to quench the reaction. Separate the repair products on a 1% agarose gel in 1X TBE buffer at 33 V overnight (12–16 h) or at 150 V for 2–3 h. Stain the gel in a solution of 1 μ g/mL ethidium bromide, and destain for 1 h in water (to reduce background fluorescence) prior to visualization on a UV light box.

4. Notes

1. The phage infection of the bacterial cells is very important for good yields of both the viral and RF DNA. The conditions for this protocol have been worked out for JM101 cells. Use of a different F' cell type may require titrating the best phage-to-cell ratio for ample DNA yield.
2. All dialysis steps, unless otherwise specified, utilize 1 L of buffer initially plus three additional changes of equal volume. Each dialysis is for a minimum of 1 h.
3. If the ratio of A_{260}/A_{280} of the purified ssDNA is below 1.8 after one CsCl gradient and subsequent phenol extraction, there is still protein contamination of the preparation. This problem can be resolved by repeating the phenol/ CHCl_3 regime or by repurifying the DNA on a second CsCl gradient.
4. It is important to be precise when weighing out CsCl. Too much or too little CsCl will alter the density of the gradient, leading to aberrant position of the DNA band.
5. Clean DNA is particularly important at the annealing step of the heteroduplex preparation. If <80–90% of the starting ds linear is converted to heteroduplex, there may be a problem with the purity of one of the starting substrates. Phenol extraction and reprecipitation of the DNA with ethanol may help this problem.
6. Although dialysis is more time-consuming than an ethanol precipitation, the overall yield of heteroduplex substrate is better when repeated precipitation of the sample is avoided.
7. The use of two smaller HAP columns is recommended over a single larger one. Some ssDNA coelutes with heteroduplex even on a larger column. Removal of this ssDNA works best with two consecutive columns.
8. After the second HAP column, dialyze the sample thoroughly against TE buffer. If the phosphate buffer is not completely removed, a fluffy white precipitate will appear during the ethanol precipitation step. Formation of this precipitate coincides with a substantial decrease in heteroduplex yield.
9. If the ExoV does not completely degrade the linear dsDNA contamination, add an additional 12–24 U of enzyme and incubate the sample at 37°C for another 90 min.
10. Occasionally, purification over two CsCl gradients will still leave small, but visible amounts of ssDNA in the heteroduplex preparation. The ssDNA comigrates on a gel with mismatch repair products and may obscure them. If this purity of substrate is not acceptable, an additional 1 mL HAP column will completely remove any ssDNA from the heteroduplex DNA.
11. The final concentration of heteroduplex can be estimated by comparison to known amounts of DNA on an agarose gel. If the heteroduplex concentration is

low (<20 ng/ μ L), the DNA can be concentrated by ethanol precipitation. Alternatively, the volume of the repair reaction can be adjusted to as much as 20 μ L, with appropriate increases in amounts of protein, cofactors and buffers.

12. To avoid loss of DNA through the extraction steps of the repair assay, care should be taken to recover as much of the aqueous phase as possible at each step. The ether extraction is important to remove residual phenol and chloroform from the DNA.
13. The amounts of restriction enzymes indicated are optimal in our hands for complete cleavage of RF DNA with no detectable cleavage of the heteroduplex control. The amounts of these enzymes can be adjusted if necessary, but the control reactions (like lanes 1, 2, and 4 of **Fig. 1B**) should be performed to confirm appropriate cutting.

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Measurement of Activities of Cyclobutane-Pyrimidine-Dimer and (6-4)-Photoproduct Photolyases

John B. Hays and Peter Hoffman

1. Introduction

The adverse biological consequences of unrepaired toxic UV-B-induced photoproducts in DNA, mostly cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6-4')-pyrimidone photoproducts [(6-4) photoproducts; (6-4)PPs], are touched on in Chapters 4, 15, and 18. Enzymes that use UV-A/blue-light energy to reverse CPDs [CPD-photolyases (PLs)] have been found in a wide variety of organisms, from bacteria to marsupials (1). In plants, enzymatic photoreactivation appears to be the predominant mechanism for repair of CPDs (2). Recently, photolyases specific for (6-4)PPs have been identified in insects (3), amphibians (4), snakes (4), and plants (5). It is often of interest to determine levels of photolyase activities quantitatively in cells in which it is difficult to assay *in vivo* repair of endogenous DNA. For example, in unfertilized eggs of amphibians, there is only a single genome, but a 10^5 -fold excess (relative to somatic cells) of ribosomes and proteins (6). If photolyase activities in extracts of such cells are to be compared, using assays based on exogenous photoproduct-containing substrates, it is necessary to achieve:

1. Photoproduct specificity.
2. Sensitivity to activities at low levels.
3. Linearity over a large activity range.
4. Resistance to interference from endogenous DNase activities in extracts, which may vary greatly from species to species.

Early CPD-photolyase assays were based on restoration of the ability of irradiated DNA to transform or transfect mutant bacteria, themselves unable to

repair or tolerate UV photoproducts, e.g., *Escherichia coli* Uvr⁻ RecA⁻ Phr⁻ (see, for example, **ref. 2** and references therein). Although often very sensitive, such bioassays are quite susceptible to degradation of substrate by endogenous DNases and are not highly reproducible. Inclusion of massive amounts of carrier DNA to swamp out DNases may markedly inhibit transformation. In general, bioassays cannot distinguish CPDs from (6-4)PPs, since neither would be repaired or tolerated in mutant bacteria. Small synthetic oligonucleotides containing defined CPDs or (6-4)PPs at specific sites have been successfully employed to purify and characterize the respective photolyases (**4**). However, the susceptibility to degradation of these (typically linear) substrates limits their usefulness in quantitative comparisons of cell-free extracts, since accurate measurement of low repair activities would be limited by the extent to which incubation times could be prolonged before substrates were significantly degraded.

The acid-hydrolysis/chromatography assay for photolyase, described in **Subheading 3.1.** below, is based on the CPD measurement technique adapted by Reynolds et al. (**7**) from the original procedure of Carrier and Setlow (**8**). It is specific for CPDs, because (6-4)PPs are destroyed by the acid treatment. The CPD-photolyase assay is virtually immune to DNase interference and has proven highly reproducible (**9**). It is fairly sensitive, because incubation times can be extended almost indefinitely. It is linear over a wide range of activities (**9**); dilution of extracts proportionally reduces repair activity, as expected, whereas dilution of interfering DNases is irrelevant. However, this assay is expensive and time-consuming.

In **Subheading 3.2.** is described a new PCR-based assay that simultaneously measures distinct CPD-PL and (6-4)-PL activities in the same sample. The circularity of the plasmid substrates and the ability to include vast excesses of carrier DNA make the PCR assay highly resistant to interference by endogenous DNases. Furthermore, an internal unirradiated plasmid control provides correction for nonspecific damage to substrate plasmids, as well as for variations in recovery of DNA from incubation mixtures. It is highly sensitive: preliminary experiments suggest that subpicogram levels of photolyase will be detectable. The ability to carry out incubations and PCRs in microtiter plates makes assay of many samples simple and convenient, once the requisite substrates are in hand.

2. Materials

2.1. Acid-Hydrolysis/TLC Assay

1. UV-irradiated [³H]-radiolabeled DNA substrate: A convenient source of radiolabeled substrate is genomic DNA extracted from *E. coli* thymine auxotrophs (*thyA*), grown in broth plus [³H] thymidine; 100 μCi/mL yields DNA with about 10⁶ cpm/μg. We extract bacterial DNA by successive proteinase-K/SDS, CTAB/

NaCl, chloroform/isoamyl alcohol and phenol/chloroform/isoamyl-alcohol treatments, and then precipitate with isopropanol (**10**). Irradiation to 1500 J/m² typically converts 4–5% of thymine to CPDs.

2. Protein extracts: The extract buffer will depend on the nature of the biological sample. For plant extracts, we use PX-Buffer: 100 mM Tris-HCl, pH 7.4, 2 mM Na₂EDTA, 10 mM dithiothreitol (DTT), and 20% glycerol, and stored at –80°C. It may be desirable to add protease inhibitors and/or increased EDTA plus EGTA (to inhibit DNases).
3. 10X PHR buffer: 500 mM Tris-HCl, pH 7.4, 10 mM Na₂EDTA, 100 mM NaCl, 100 mM DTT.
4. 20% (w/v) Trichloroacetic acid (TCA) (ice-cold).
5. Carrier DNA: salmon sperm or similar crude DNA, sonicated to reduce size to roughly 5–10 kb, 5 mg/mL stock.
6. Silica-gel plastic-backed thin-layer-chromatography (TLC) plates (Baker-flex IB2-F, VWR, Seattle, WA), 20 × 20 cm², previously washed by mock chromatography in distilled H₂O. Draw light pencil lines 2 and 12 cm from the bottom edge, and parallel with it, to mark the origin and ending positions, and 9 2-cm vertical lanes (bordered by 1 cm).
7. TLC development buffer: ethyl-acetate:*n*-propanol: H₂O, 4:1:2. Prepare by mixing in a separatory funnel, allow to separate for at least 4 h, and then recover the organic phase. Make fresh monthly and store in glass-stoppered glass bottles.
8. Stop solution: 0.2% sodium dodecylsulfate (SDS), 30 mM Na₂EDTA, pH 8.0.
9. Formic acid (>98% grade).
10. Liquid nitrogen.
11. Thick-wall ignition tubes, 40 × 100 mm (Corning, catalog no. 401530).
12. Adapters for ignition tubes (1.8-cm id), to fit a Beckman GA-10 centrifuge rotor.
13. 20-W blue or “Black” (375 nm) light source, and lamp with red or gold bulbs.
14. Glass cutter.
15. Diagonal wire cutters.
16. Drying manifold, suitable for air-drying nine or more ignition tubes.
17. Scintillation vials.
18. Water-compatible scintillation fluid, such as NEN-989 (New England Nuclear, Boston, MA).
19. Beckman GA-10 rotor and GPR centrifuge.
20. Access to oxyacetylene torch.
21. Oven (heated to 185°C).
22. Chromatography developing chamber, suitable for 20 × 20 cm² TLC plate.
23. 1.9-cm Transparent tape (e.g., Scotch Magic tape).
24. Liquid-scintillation spectrometer.

2.2. PCR-Amplifiability Assay

1. Items 1–3 and 8 of **Subheading 2.1**.
2. CPD-substrate plasmid (pCPD1, 3.0 kb): Construction of pCPD1 and pSFA1 (*see item 3 below*) will be described in detail elsewhere. Briefly, pCPD1 was derived

from pBSII S/K(–) (Stratagene, San Diego, CA), itself selected because of a greater-than-average number of polythymine runs, likely hot spots for induction of CPDs by UV light. We modified pBSII S/K(–) by converting its *EcoRV* restriction site to *NcoI*, and inserting a synthetic TTT/TTTT-rich 53-mer into its *NaeI* site (within the 2513-bp region amplified by the specific primers [see **item 5** below]). The small fraction of (6-4)PPs in UV-irradiated pCPD1 presents no problem in most applications. If no (6-4)-photolyase or other activity that repairs (6-4)PPs is present, it is irrelevant, since CPD photoreactivation is measured as the net decrease in apparent blocking lesions. Any repair of (6-4)PPs that does occur may be measured unambiguously as repair of irradiated plasmid pSFA1 present in the same reaction mixture, and the latter value subtracted from the value for repair of plasmid pCPD1 (see **Note 1**).

3. (6-4) Photoproduct-substrate plasmid (pSFA1, 4 kb): To construct plasmid pSFA1, we modified pBR322 (**11**), in which the frequency of TC sequences in contexts expected to enhance (6-4)PP frequency (see, for example **ref. 12**) appeared relatively high, by converting the *MscI* restriction site to *NcoI*, deleting DNA between the *SspI* and *ScaI* sites, and inserting a TC-rich synthetic 51-mer into the *BsaAI* site. The deletion and insertion resulted in a 2282-bp target for PCR amplification using the primers indicated below (**item 5**).
4. Recovery/degradation control plasmid (pREP4, 3.1 kb): Unirradiated pREP4 (Qiagen) is included in repair reactions with irradiated pCPD1 and pSFA1, so that in the subsequent assay reaction, its PCR product, electrophoretically resolvable from products templated by the latter two plasmids, provides internal normalization for degradation or loss during repair and processing. The primers indicated below template a PCR product of 2843 bp.
5. PCR primers: The primers below have been designed for simultaneous amplification of the three plasmids described above, in the same “multiplex” reaction, yielding respective PCR products resolvable from one another by gel electrophoresis:
 - pCPD1-for, 5' TTGGCGTAATCATGGTCATAGC
 - pCPD1-rev, 5' TAAAGAACGTGGACTCCAACGTC
 - pSFA1-for, 5' AACAGGCAGACATCTGTAATCGC
 - pSFA1-rev, 5' ATTGTTAGATTTTCATACACGGTGCC
 - pREP4-for, 5' TTGATGGTGGGTAAACGGCGGGATA
 - pREP4-rev, 5' GATGCTCTTCGTCCAGATCATCCTGA
6. Carrier DNA: Because PCR technology has been developed to amplify as few as one sequence in a genome of 10^9 bp or more, virtually any amount of nonspecific carrier DNA may be added to the repair reaction to protect substrates against (divalent-cation-independent) DNases. Inhibition of repair enzymes by nonspecific binding may be a concern. We currently use salmon sperm DNA, ultrasonically degraded to an average size of 5 kb.
7. Microtiter dishes and accessories: The method is described below (**Subheading 3.2.**) in the context of multiple (up to 96) assays, as might be encountered in assaying chromatographic fractions or several repetitions of a series of biological samples, because this makes fullest use of the advantages of the PCR assay.

However, the technique can be applied equally well to a small number of samples in microcentrifuge tubes. We use 96-well 200- μ L microtiter plates, 96-well 200- μ L PCR plates, a 96-well vacuum manifold, and an 8-well multichannel pipeter.

8. Phaselock Gel (5-Prime $\rightarrow$ 3-Prime, Inc., Boulder, CO) (optional).
9. $MgCl_2$ stock, 25 mM.
10. *Taq* DNA polymerase and 10X *Taq* buffer (Perkin-Elmer, Norwalk, CT).
11. Mixture of four dNTPs, 2 mM each.
12. [^{32}P]dCTP, 3000 Ci/mmol.
13. *Nco*I restriction endonuclease and 10X buffer (New England Biolabs, Beverly, MA).
14. TE buffer: 10 mM Tris-HCl, pH 7.5, 1 mM Na_2EDTA .
15. G-50 minicolumn plate, 96 wells. Prepare as follows: Puncture a hole in the bottom of each (200- μ L) well in a PCR-reaction plate (the plastic is thinner and the wells deeper than in standard microtiter plates) with a 21-gage needle. Place a small piece of sterile cotton in the bottom of each well, and fill completely with G50-Sephadex (Fine) beads (Pharmacia, Piscataway, NJ) slurried in TE buffer. Using a 96-well vacuum manifold attached to the bottom of the tubes, or tubing connected to each tube and to a trap/vacuum system, draw out enough liquid to leave a semidry G-50 column filling about 1/2 of the well. Refill with additional G-50 slurry while applying vacuum to the bottoms of the tubes. The final column should fill 3/4 of the well. Stack the plate of minicolumns onto a second plate, and centrifuge both at 1000g for 10 min. Stack the G-50 column plate onto a new plate.
16. Sterile H_2O .
17. Phenol (containing 0.1% 8-hydroxyquinoline):chloroform:isoamyl-alcohol, 25:24:1.
18. TBE buffer for gel electrophoresis: 90 mM boric acid, 90 mM TRIZMA base (Sigma, St. Louis, MO), 2 mM Na_2EDTA .
19. LE-agarose (FMC Corp., Rockland, ME).
20. Vertical gel-electrophoresis apparatus with circulating cold-water cooling, e.g., Bio-Rad Protean II.
21. Whatman 3MM paper.
22. Dryer for slab gels, with vacuum manifold.
23. Beckman GP centrifuge, with TGH 3.7 swinging-bucket rotor, equipped with microtiter plate holders.
24. 96-Well-block thermocycler, e.g., Stratagene Robocycler 96.
25. PhosphorImager (e.g., Molecular Dynamics).

3. Methods

3.1. Acid-Hydrolysis/TLC Assay

3.1.1. Photoreactivation

Steps 1–3 below are ideally performed under red or gold light.

1. Mix at least enough UV-irradiated DNA substrate to supply $2.5\text{--}3 \times 10^5$ cpm (typically 200–800 ng) with 5 μ L of 10X PHR buffer in the wells (200 μ L) of a

96-well microtiter dish. More substrate may be needed to ensure enzyme saturation (*see Note 2*).

2. Prepare an unirradiated DNA control to carry through the entire procedure.
3. To each well, add desired amounts of protein extract (*see Note 2*), and extract buffer to make the final volume up to 50 μL .
4. Incubate the samples under blue or “black” light for 60 min at room temperature, or for other times appropriate for the amount of activity (*see Note 2*). Dark controls may be incubated in a separate microtiter dish wrapped in foil.
5. Add 1 vol of stop solution.
6. Transfer quantitatively to ignition tubes.

3.1.2. Acid Hydrolysis

1. Add 5 μL (25 μg) of carrier DNA, mix well, then add 1 vol of cold 20% TCA, and incubate for 10 min on ice.
2. Centrifuge the ignition tubes, in adapters in a GA-10 rotor, at 5000g for 10 min at 4°C.
3. Decant the (radioactive) supernatant, cover the pellet with ice-cold 95% ethanol, centrifuge as above, and again decant.
4. Dry the pellet with a gentle stream of air under a drying manifold.
5. Add 200 μL of formic acid (98%) and centrifuge briefly.
6. Install the tubes at an angle of 45° in an insulated clamp. Seal with an oxyacetylene torch, heating the bottom side about 1–2 cm from the lip of the tube, working around to the top as the glass softens, then pulling out the lip with tongs to about a 5-cm neck; cool for at least 10 min. At this point, the tube, while constricted, should still be open to the air. Briefly heat the neck to seal it off about 1 cm from the tube proper; make sure a small glass ball forms at the end of the neck. Be aware that sealed tubes have been reported to burst occasionally; use appropriate safety equipment. *See also* refs. (7) and (8).
7. Place the sealed tubes in a heat block in a 185°C oven for 45 min.
8. Remove the tubes, and let cool to room temperature in a heat-resistant rack.
9. Score a ring around the tubes, about 2/3 of the distance from the bottom (but still in the constant-diameter portion).
10. Centrifuge briefly to collect the liquid, which may appear brown if the protein concentration was high (*see Note 3*).
11. Immerse the tube in liquid nitrogen until the liquid freezes solid.
12. In a fume hood (formic-acid vapor is acrid and toxic), cut off the glass ball at the end with diagonal cutters, restraining the ball to keep it from popping away. Keep the solution frozen to reduce the pressure released by cutting.
13. Break the tubes cleanly and safely at the score lines, using two short pieces (25 cm) of heavy-duty metal tubing just large enough to fit smoothly around the glass ignition tube. Place the tube in one metal piece so the score line is just exposed, slide the other down to meet it (completely hiding the glass tube), and then flex the two pieces away from the worker. **Important: Do not** attempt this without first releasing the pressure, as in **step 12** above.

14. With the open tubes still in the hood (in a 55°C heat block if speed is important), blow a steady gentle stream of air, until the pellet (clear to very brown) is dry, and the very acrid fumes are completely dissipated.
15. Resuspend the pellet in 20 μ L of H₂O using a vortex mixer.

3.1.3. Thin-Layer Chromatography and Analysis

1. Carefully spot samples along the origin line of the TLC plate, 7 μ L/application, allowing the spots to dry completely each time. Then place the plate in the developing chamber, just after adding 100 mL of fresh developing solution.
2. Develop the plate until the front has moved 10 cm from the origin line (to the 12-cm pencil line).
3. Hang the plate in a fume hood to dry. Bands corresponding to the respective bases can now be visualized using 365-nm light, if the TLC plate incorporates a fluorescent indicator.
4. Overlay the lanes with 1.9-cm transparent tape, press down firmly, and partition the lanes into 0.5-cm fractions with light pencil lines.
5. Use a single-edge razor blade to separate the lanes by cutting through the chromatography matrix, but not the plastic backing. The strips should extend from 1 cm below the origin to 1 cm above the final development front.
6. Grasping the plate at the extreme top and bottom, flex it across the edge of a lab bench, working from bottom to top, to separate the matrix from the backing, yielding nine 2 cm $\times$ 12 cm strips.
7. Cut off individual 0.5-cm fractions, and place in (disposable) scintillation vials, with 250 μ L of H₂O and 3.5 mL of scintillation fluid.
8. Determine the radioactivity profiles in a liquid scintillation counter, and identify the CPD peak by comparison with the unirradiated DNA control. Subtract a background corresponding to the average of the cpm in the next adjacent "valley" fraction on each side of the CPD peak. Divide by total cpm in all of the fractions from each lane, thus expressing the data as a fraction of total [³H] thymine in CPDs [not distinguishing among T<>T, and the T<>U and U<>T hydrolysis products of T<>C and C<>T CPDs).
9. Plot the fraction of [³H] CPDs as a function of extract concentration, ideally from duplicate assays at each concentration. The slope of the linear portion of the curve yields the photolyase specific activity, usually expressed in CPDs removed/h/ μ g protein (see **Note 2**).

3.2. PCR-Amplification Assay

3.2.1. Photoreactivation and Sample Recovery

1. Repair reactions are essentially as described in **Subheading 3.1.1.**, except that only about 50, 25, and 10 ng of plasmids pCPD1, pSFA1, and pREP4 are needed. Enough carrier DNA to ensure satisfactory recovery of undegraded substrate, without inhibiting repair reactions (typically 5 μ g), should be added, and incubations should be in a 200- μ L well microtiter dish.

2. Add 0.1 vol of 10X stop solution to terminate the reaction and use a multichannel pipeter to transfer mixtures to a 96-well PCR reaction plate, whose wells already contain 55 μ L phenol:chloroform:isoamyl-alcohol (and Phaselock Gel, *see step 4* below). Emulsify by pipeting up and down about 10 times.
3. Centrifuge the microtiter plates in a Beckman GP Centrifuge (GH 3.7 rotor) at 500g for 10 min at room temperature. Transfer the supernatants to the corresponding wells of the G50-minicolumn plate using the multichannel pipeter. It is more important to avoid the phenol layer than to recover all of the aqueous layer.
4. Sediment the samples through the columns into the lower plates for 10 min at 1000g. About 50 μ L should be recovered. If the solutions are not too dense (not from sucrose gradients, for example), recovery can be enhanced by including 80 μ L of Phaselock Gel with the phenol:chloroform:isoamyl alcohol in the wells.

3.2.2. Preparation and PCR Amplification

1. Digest 10- μ L samples, in microtiter wells, with 5 U of restriction endonuclease *Nco*I, which cuts each substrate plasmid once, for 2–3 h. (In our hands, supercoiled plasmids inefficiently template PCR amplification of products that are nearly as large as the plasmids themselves.)
2. Prepare 96-well PCR plates with 27 μ L of PCR cocktail (final 1X *Taq* buffer, 3 U of *Taq* polymerase, 1.5 mM $MgCl_2$, 120 μ M dNTPs, 1 μ Ci [^{32} P]dCTP, 2.5 μ M of each of the (six) primers, and H_2O as necessary).
3. Use the multichannel pipeter to add 3 μ L of *Nco*I digest to the respective wells.
4. Amplify the DNA in a thermocycler using the following cycle:
 - a. One-time 94°C soak, 3 min.
 - b. Repeated 94°C denaturation, 45 s.
 - c. Repeated 52°C annealing, 45 s.
 - d. Repeated 72°C extension, 3 min.

We repeat **steps b–d** for 12 cycles, to remain within the exponential amplification range (*see Note 4*). (Conditions are optimal for the Stratagene thermocycler, and the templates and primers described, and should be adjusted empirically.)

3.2.3. Electrophoretic Analysis

The procedure below assumes that analysis of electropherograms will be by measurement of radioactivity rather than by ethidium bromide fluorescence (*see Note 4*).

1. Prepare a 0.6% agarose/TBE vertical gel, 1 mm $\times$ 20 cm $\times$ 20 cm, with approx 40- μ L wells.
2. Add 5 μ L of 10X Stop solution to each finished PCR, and load 20 μ L into the agarose-gel wells.
3. Electrophorese at 50 V for 16–18 h, until the bromphenol blue dye enters the lower buffer chamber (along with unincorporated [^{32} P]dCTP, which must be handled appropriately).

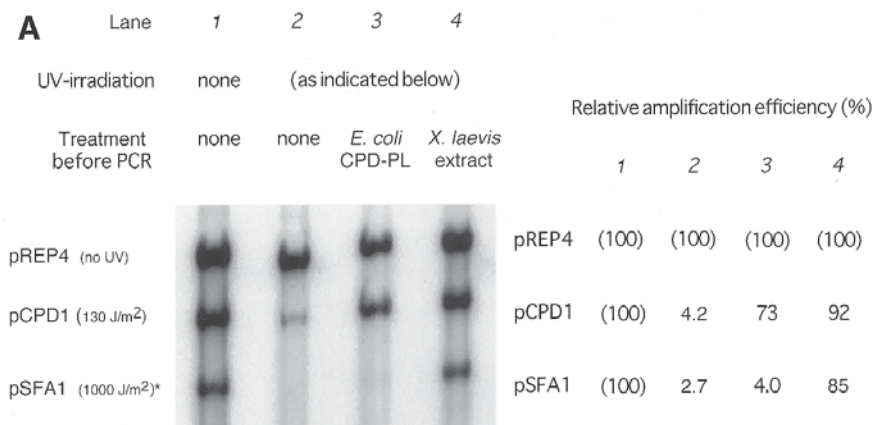
4. Carefully remove the gel from the glass plate onto a piece of Whatman 3MM paper, overlay with Saran Wrap, and dry in gel dryer.
5. Place the dry gel in a Phosphorimager cassette, and expose as necessary (typically 30 min). When sufficient image has accumulated, remove the gel and scan the cassette with the phosphorimager. (See **Fig. 1A**.)
6. To analyze, we generate a full line scan using the Molecular Dynamics Image Quant software package. The line scan trace is then used to establish by eye the background values at the leading and trailing edges of each peak. If these are significantly different, use an average value. The backgrounds can vary significantly from lane to lane and peak to peak, and must be determined individually (see **Note 5**). The software can be used to calculate the net area under each peak. (See **Fig. 1B**.)

3.2.4. Calculations

The relative amplifiabilities of the substrate plasmids (before and after photoreactivation) emerge from a double normalization. First, the net areas under the pCPD1 and pSFA1 substrate peaks are divided by the area of the pREP4 recovery-control peak in each lane. Second the ratios for all of the lanes corresponding to UV-irradiated (repaired) DNA are normalized by the same ratios for the unirradiated DNA mock-repair samples. The normalized ratios (r) then yield the average number (b) of blocking lesions (photoproducts) per plasmid, $b = -\ln(r)$. For the analyses shown in **Fig. 1**, irradiation of pCPD1 at 254 nm to 130 J/m² reduced relative PCR-amplifiability of the 2.5-kb target region to 4.2% (3.17 Poisson-distributed PCR-blocking lesions, presumably mostly CPDs), and irradiation of pSFA1 to 1000 J/m² plus exhaustive photoreactivation with purified CPD-photolyase (kind gift of A. Sancar) reduced amplifiability of the 2.3-kb target to 2.7% (3.6 blocking lesions, presumably mostly [6-4]PPs) (**Fig. 1A**, lane 2). Treatment of a mixture containing the two substrates plus plasmid pREP4, with *E. coli* photolyase increased pCPD1 amplifiability to 73% (0.32 blocking lesions) and that of pSF4 to only 4.0% (3.2 lesions), confirming the nature of the respective blocking lesions (**Fig. 1A**, lane 3). Treatment of the same substrate mixture with an extract from *Xenopus laevis* oocytes (9) restored the respective amplifiabilities to 92 and 85% (0.1 and 0.16 blocking lesions) (**Fig. 1A**, lane 4), confirming the presence of both photolyases (4,9). (see **Note 6**.)

4. Notes

1. Instead of irradiating pCPD1 at 254 nm, and taking account of the fact that ~10% of the photoproducts are (6-4)PPs, a substrate essentially free from (6-4)PPs may be prepared by treating pCPD1 irradiated at 254 nm with purified (6-4)-photolyase, if available, or irradiating pCPD1 at 313 nm in the presence of a photosensitizer, such as acetophenone, which treatment induces almost exclusively thymine-thymine CPDs (13).



* Exhaustively treated with *E. coli* CPD-photolyase (PL) after irradiation

Fig. 1. PCR assays of CPD- and (6-4)-photolyase activities. Plasmids pCPD1 and pSFA1 were respectively irradiated at 254 nm to 130 J/m², or irradiated to 1000 J/m² and treated exhaustively with purified *E. coli* photolyase. Mixtures containing 25 and 50 ng, respectively of these plasmids, plus 10 ng of plasmid pREP4 and 1 µg of carrier DNA, were photoreactivated for 120 min using a soluble protein extract from *X. laevis* oocytes (about 150 µg), in a total volume of 50 µL, as described in **Subheading 3.2.1**. Parallel samples were treated for 120 min with purified *E. coli* CPD-photolyase (1 µg). (A) Phosphorimages of electropherograms of PCR products. Aliquots (3 µL) of reaction mixtures were analyzed by linearization using restriction endonuclease *Nco*I, PCR amplification using [³²P] dCTP, and electrophoresis and phosphorimaging, as described in **Subheadings 3.2.2.** and **3.2.3.** PCR products shown were templated by the following mixtures: lane 1, unirradiated plasmids; lane 2, photoproduct-containing pCPD1 and pSFA1 (irradiated/treated as above) plus unirradiated pREP4; lane 3, plasmid mixture as in lane 2, photoreactivated with *E. coli* photolyase; lane 4, mixture as in lane 2, photoreactivated with *X. laevis* oocyte extract. Relative amplification efficiencies equal intensities of PCR-product bands corresponding to respective irradiated/repared plasmids (lanes 2–4) divided by intensities for unirradiated plasmids (lane 1), and normalized by ratios of pREP4 intensities in lanes 2–4 to pREP4 intensity in lane 1. Values were determined from areas of respective peaks in corresponding line scans (B).

- The amounts of DNA substrate and extract protein, and the time of incubation, are dictated by two considerations. First, there should be at least enough photoreactivation to reduce the fraction of [³H] thymine in CPDs by a reproducibly detectable amount, from 4 to 5% down to ~3% or less, but not so much that almost all CPDs are removed (at least 1% remaining). Operationally, it is simplest first to determine an appropriate incubation time and range of concentra-

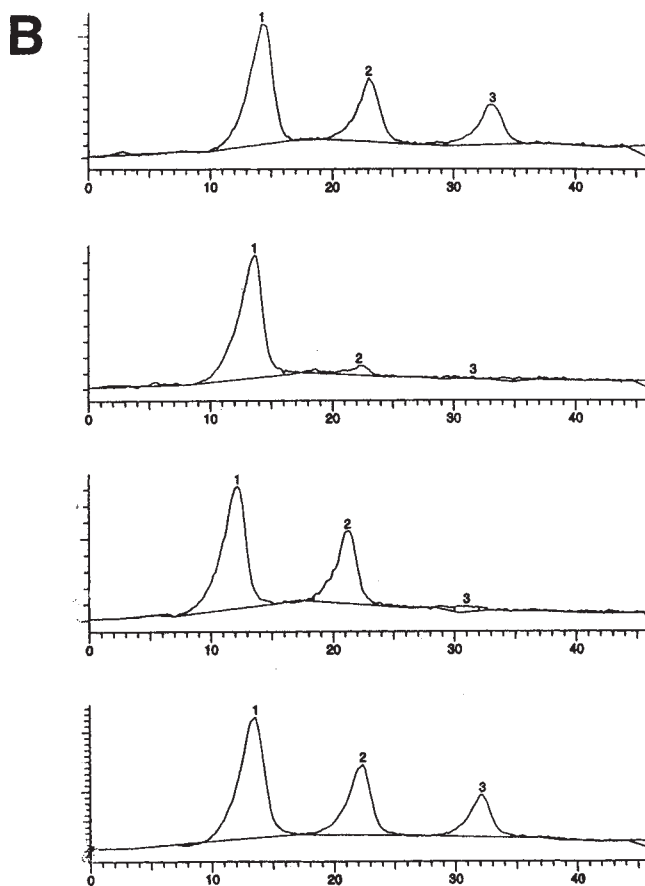


Fig. 1(B). Line scans of phosphorimages (see step 6 of Subheading 3.2.3., and Note 5). Traces correspond to lanes in Fig. 1(A). Baselines are assigned by eye at the next valley level. (Machine-assigned baselines tend to vary dramatically and to be dependent on signal intensity.) Peaks 1, 2, and 3 represent the intensities of amplified products from pREP4, pCPD1, and pSFA1, respectively. Y-axis values are automatically scaled and assigned by the phosphorimager software based on the greatest peak height and, therefore, vary from trace to trace. X-axis values represent the distance (in mm) along the lane trace from an arbitrary starting point.

tions in a preliminary experiment, and perform the final determination at a series of concentrations, such that CPDs removed will be directly proportional to protein concentrations for at least 3–4 points. Second, the DNA concentration should be well in excess of the apparent K_m for the particular photolyase. Operationally, this is verified by showing that incubation of the amount of extract correspond-

ing to the highest linear repair point (highest decrease in [^3H] thymine in CPDs), with double the previous amount of UV-irradiated DNA substrate, results in repair of the same absolute amount of [^3H] CPDs as previously (now 1/2 the previous reduction in relative fraction of [^3H] thymine in CPDs).

3. Avoid using more than 200 μg protein in assays, since chromatographic separation is less clean.
4. Implicit in the assay is the assumption that the amount of final PCR product is directly proportional to the starting amount of (undamaged) template (substrate plasmid), for all samples. This requirement is fulfilled as long as the amount of product increases exponentially with the number of cycles, again for every sample. In our hands, this “log-linear” product accumulation falls off after 15 cycles or so, typically before DNA can be measured accurately by ethidium bromide fluorescence. The electrophoresis/phosphorimaging approach described offers the advantages of internal normalization for recovery and minimum background. Alternatively, PCR products may be measured as acid-precipitable radioactivity. This requires four separate PCR assays for a particular (three-plasmid) repair reaction: three PCR amplifications with each pair of respective primers, plus no-primer and/or no-template background-control reactions.

In any case, log-linear amplification of representative samples should be verified in two ways: first, by measuring product after successive rounds of amplification, and choosing a standard number of cycles safely less than the number of cycles at which a plot of log (product) vs number of cycles falls below a straight line, and second, by showing that product increases linearly with amount of template initially added, in a series of standard-cycle-number amplifications.

5. It is critical to determine the background radioactivity from the graphical profile. In our experience, other methods—counting a box elsewhere in the lane, counting around the border of the sample box, for examples—can yield quite different values, so that the net value for the signal becomes almost arbitrary.
6. We now find the PCR assay to be in practice unsuitable for *initial-rate* measurements over a wide range of substrate concentrations, because the “product” signal (increased PCR yield) is strong relative to the noise level only if the number of PCR-blocking lesions is on average substantially reduced for *all* of the substrate plasmids. At high substrates (in excess of the Michaelis constant, K_m), this degree of reaction may require high enzyme/extract concentrations or long incubation times that are impractical. At low substrate concentrations, removal of a significant number of lesions from all plasmids affects the reaction velocity, so true initial rates cannot be measured. However, the PCR assay is ideal for following the entire reaction time course, because inhibition by product (lesion-free DNA in this case) is negligible and, with low amounts of substrate (typically 2–10 ng), repair can go to completion in a time too short for significant loss of enzyme activity. At very low concentrations, substrate (DNA-lesion) levels can decay by strict first-order kinetics; the slope of a plot of *in* [average number of lesions (from the PCR data)] against time yields the catalytic efficiency (V/K_m). Cata-

lytic efficiency is actually the biological relevant parameter for comparisons of photolyase levels among different tissues or different organisms, because in nature, solar UV-B light typically produces low photoproduct levels in cells. Nevertheless, if initial substrate concentrations are high enough above the first-order range, analysis of the reaction time course using the integrated rate equation yields K_m and the maximum velocity V separately (14).

Acknowledgment

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A Dot Blot Immunoassay for UV Photoproducts

Shirley McCready

1. Introduction

The dot-blot method described here can be used to measure repair of pyrimidine-pyrimidone 6-4 photoproducts ([6-4]PPs) and cyclobutane pyrimidine dimers (CPDs) in total genomic DNA from any organism. The DNA does not have to be especially intact nor is it necessary to have any sequence information. Although the protocol given below is for measuring repair in budding yeast, the method has been successfully used to measure repair in fission yeast, human cells, archaebacteria, *Streptomyces*, *Aspergillus*, and plants (1-3; and McCready, unpublished). The assay is sufficiently sensitive to measure damage induced by 10 J/m² of UV-C with ease and could be used for lower doses.

To use the method, it is necessary to raise polyclonal antiserum to UV-irradiated DNA. The antiserum must be characterized for its ability to recognize damage that can be photoreactivated by *Escherichia coli* photolyase (CPDs) and damage that cannot (predominantly [6-4]PPs and the Dewar isomer of [6-4]PPs [4,5]). An antiserum containing activities against CPDs and (6-4)PPs can be used to measure total lesions. Alternatively, it can be used to measure each type of photoproduct individually by destroying one or the other lesion in the DNA before carrying out the assay. (6-4)PPs can be destroyed by treating DNA samples with hot alkali before applying DNA to the blotting membrane. CPDs can be destroyed in DNA after it has been applied to the blot by treating the entire blot with *E. coli* photolyase.

Cells are irradiated and samples are harvested immediately and after suitable incubation periods. DNA can be extracted from the cells by a variety of procedures—commercially available kits, or by phenol or chloroform-phenol extraction. It is of crucial importance to equalize the amounts of DNA in samples from the different time-points, and this is best done by running ali-

quots on an agarose gel and estimating relative amounts by densitometry. Concentrations must be adjusted and checked on gels for as many times as necessary until the DNA concentrations are uniform. Each DNA sample is then divided into two, and one half is treated with hot alkali to destroy (6-4)PPs. Dilution series of the samples are then applied to duplicate dot blots. One blot is exposed to a crude preparation of photolyase and illuminated with visible light to destroy CPDs. The blots are then exposed to polyclonal antiserum, then to a biotinylated secondary antibody, and then to an alkaline phosphatase-conjugated avidin. Nitroblue tetrazolium is used as substrate so that a blue color stains the DNA dots containing UV lesions. Over a certain range, the amount of blue color is proportional to the amount of damage. Blots contain their own in-built calibration curves, namely, the dilution series of the time-zero samples. The amount of damage remaining in postincubation samples is quantitated by densitometry and reference to the time-zero dilution series.

2. Materials

All media and aqueous buffers should be sterilized by autoclaving.

2.1. Production of Polyclonal Antiserum

1. Isotonic saline: 0.15 M NaCl, pH 7.0.
2. Calf thymus DNA (e.g., Sigma, Poole, UK): dissolve at 1 mg/mL in isotonic saline.
3. Methylated bovine serum albumin (MBSA): dissolve at 2 mg/mL in water and add an equal quantity of 2X isotonic saline (final concentration 1 mg/mL in isotonic saline).
4. Poly[dA] · poly[dT] (Sigma, Poole, UK).

2.2. Preparation of Crude Photolyase

1. Luria Broth with tetracycline (20 µg/mL final concentration).
2. Isopropylthio-β-D-galactopyranoside (IPTG): 0.2 g/mL in water (840 mM).
3. Lysis buffer: 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 10 mM β-mercaptoethanol.
4. Storage buffer: 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM dithiothreitol (DTT), 50% glycerol.
5. *E. coli* strain PMS 969 [PHR1] (from Aziz Sancar; (6)).

2.3. Repair Experiments and DNA Isolation

1. YEPD medium: 1% yeast extract, 1% peptone, 2% dextrose.
2. 10X YEPD: 10% yeast extract, 10% peptone, 20% dextrose.
3. 1 M sorbitol.
4. Zymolyase: Zymolyase 20T (ICN Biochemicals, UK) dissolved in water at 10 mg/mL.
5. Tris-EDTA (TE): 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
6. 10% SDS: 10% (w/v) Sodium dodecylsulfate in water.

7. Phenol-chloroform: 50 mL of TE-equilibrated phenol, 50 mL of chloroform, 2 mL of isoamyl alcohol.
8. 3 M sodium acetate, pH 5.2.
9. RNase A solution (DNase-free) (Sigma, Poole, UK, cat. no. R 4642).
10. Ethanol.

2.4. Preparation and Processing of Dot Blots

1. DNA containing only thymine dimers: Irradiate herring sperm DNA (0.1 mg/mL) in 10 mM acetophenone in an open Petri dish with midwave UV (e.g., using a Westinghouse FS20 sun lamp). Under these conditions the only detectable photo-products produced are thymine dimers (7).
2. 1 N NaOH. Always make fresh.
3. Neutralizing solution: 3 M potassium acetate in 5 M acetic acid.
4. Nitrocellulose membrane (e.g., Schleicher & Schuell, Germany) (*see Note 1*).
5. 1 M ammonium acetate.
6. 5X SSC: 0.75 M sodium chloride, 0.075 M sodium citrate, pH 7.0.
7. 1% Gelatin: warm to dissolve the gelatin.
8. Carrier DNA: a scaled-up crude DNA preparation from yeast, prepared as in **steps 1–6 of Subheading 3.4**.
9. Phosphate-buffered saline (PBS): 20 mM sodium phosphate, 150 mM NaCl.
10. PBNT: PBS containing 0.5% normal goat serum, 0.5% bovine serum albumin (BSA), 0.05% Tween-20.
11. PBX: PBS containing 0.1% Triton X-100.
12. Biotinylated antirabbit antiserum and alkaline phosphatase-conjugated ExtrAvidin (ExtrAvidin® Alkaline Phosphatase staining kit, Sigma EXTRA-3A) (*see Note 2*).
13. Tris-buffered saline (TBS): 50 mM Tris-HCl, pH 7.4, 150 mM NaCl.
14. Alkaline phosphatase buffer: 100 mM NaCl, 5 mM MgCl₂, 100 mM Tris (should be pH 9.5 without need of adjustment).
15. Alkaline phosphatase substrate: Nitro blue tetrazolium, 5-Bromo-4-chloro-3-indolylphosphate (Gibco BRL, Paisley, UK) (*see Note 2*).
16. PBS-EDTA: PBS containing 0.75% EDTA.
17. Scanning densitometer with image analysis instrumentation (e.g., a Bio-Rad GS-670 Imaging Densitometer with Molecular Analyst image analysis software).

3. Methods

3.1. Preparation and Characterization of the Polyclonal Antiserum

The antiserum is raised in rabbits, following the protocol described by Mitchell and Clarkson (4) (*also see* Chapter 14).

1. Phenol-extract and ethanol-precipitate calf thymus DNA. Dissolve in isotonic saline at a concentration of 1 mg/mL.
2. Irradiate the DNA in an open Petri dish on ice, giving a total dose of 100 kJ/m².

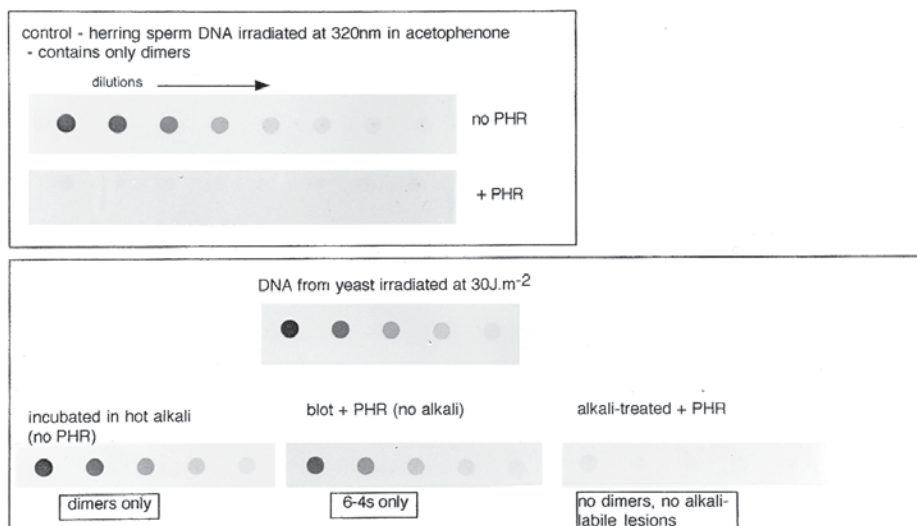


Fig. 1. Strip tests for polyclonal antiserum. The control DNA (top panel) contains only CPDs, which are completely removed by incubating the blot in photolyase (PHR) under visible light illumination (photoreactivation). Yeast DNA incubated in hot alkali (lower left) contains only CPDs, which are completely removed if the blot is treated with photolyase (lower right). Photolyase treatment alone removes CPDs (lower middle) and leaves alkali-labile sites, which are principally or entirely (6-4)PPs.

3. Readjust the concentration of the DNA to 0.4 mg/mL by isotonic saline as appropriate.
4. Prepare 1 mL of immunogen by mixing 0.5 mL of irradiated, heat-denatured DNA with 0.5 mL of MBSA. Mix well and filter-sterilize.
5. For the first injection, emulsify 1 mL of immunogen with 1 mL of complete Freund's adjuvant. Give four subsequent injections every 2 wk using incomplete adjuvant. Two weeks after the last injection, administer a booster of 200 μ g of poly[dA] · poly[dT] DNA irradiated with a dose of 250 kJ/m². Preimmune serum and test bleeds taken after each injection must be checked for activity. Harvest the antiserum 2 wk after the booster. The exact details of this protocol must be approved and possibly modified according to local rules for animal handling.
6. Test bleeds: Prepare test strips by applying a dilution series of denatured herring sperm DNA, which has been irradiated with UV-C at 50 J/m² to dot blots in the same way as for the repair assay (*see Subheading 3.5.*). Process the test strips in exactly the same way as for the repair assay (*see Subheading 3.6.*). The activity of the antiserum against total lesions and nondimer photoproducts should be monitored (**Fig. 1**).

3.2. Preparation of Crude Photolyase

This method is based on the first part of the purification procedure for photolyase described by Sancar et al. (7).

1. Grow *E.coli* [PHR1] in 1 L of Luria broth containing tetracycline (25 mg/L) to $OD_{600} = 1.0$ -1.1. Add IPTG to 0.5 mM. Grow for a further 12 h.
2. Harvest the cells by centrifugation and wash in lysis buffer.
3. Resuspend in 20 mL of ice-cold lysis buffer. Divide into three, and sonicate (four 30-s pulses on ice). Keep the lysate cool.
4. Spin at 16,000 rpm in an SS-34 rotor (31,000g) at 4°C for 20 min.
5. Spin the supernatant in an ultracentrifuge at 35,000 rpm in a Ti 50 rotor (120,000g) at 4°C for 1 h.
6. To 20 mL of supernatant, add 8.6 g of ammonium sulfate, slowly, over a 1-h period, keeping on ice and swirling to dissolve well.
7. Spin down the yellow precipitate, in a sterile Corex tube, at 8000 rpm in an SS 34 rotor (8000g) for 30 min at 4°C.
8. Dissolve the precipitate in 5 mL of ice-cold storage buffer. Add 100- μ L aliquots to precooled 0.5-mL microcentrifuge tubes and store at -70°C.
9. The photolyase preparation should be tested for photoreactivating activity on test strips (Fig. 1).

3.3. Repair Experiment

1. Irradiate midlog-phase cells in sterile water at a cell density of $1-2 \times 10^7$ /mL using a dose of 50 J/m². The cells should be irradiated as a 0.5-cm suspension in an open plastic tray. You will need 30 mL of cell suspension for each time-point.
2. Immediately after irradiation, take a 30-mL sample and add to 30 mL of ice-cold ethanol. This will serve as the time-zero sample.
3. Divide the remaining suspension into 30-mL aliquots. Add 3 mL of 10X YEPD to each, and incubate at 28°C with gentle shaking. For each time-point, add one of the 30-mL cultures to 30 mL of ice-cold ethanol, and keep on ice for 5 min before harvesting by centrifugation at 15,000 rpm (SS-34 rotor, 27,000g) for 10 min.
4. Resuspend the cells in 1 mL of TE. Transfer to a microcentrifuge tube. Wash the cells in TE, and then in 1 M sorbitol.

3.4. DNA Extraction

A commercial kit for genomic DNA isolation (e.g., Nucleon BAC1 kit, Nuclear Biosciences, UK) can be used. Alternatively, DNA can be extracted by phenol-chloroform extraction as follows:

1. Resuspend the cells in 500 μ L of 1 M sorbitol, and add 25 μ L of zymolyase to convert the cells to spheroplasts. Check the cells under the microscope—spheroplasts are round and dark under phase contrast, and they will swell and burst in water.

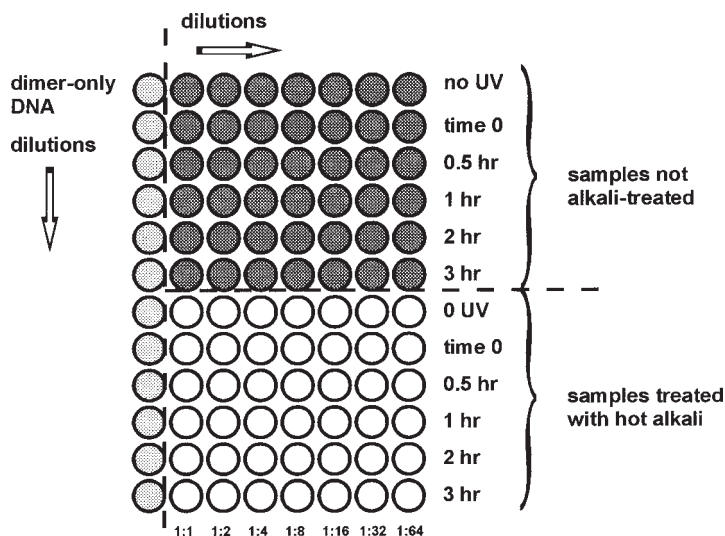


Fig. 2. Layout of the dot blots. Doubling dilutions in 1 *M* ammonium acetate are set up in microtiter plates, and samples transferred to a blot in the array illustrated.

2. Spin down the spheroplasts in a microcentrifuge at 12,000*g* for 3 min.
3. Resuspend the spheroplasts in 500 μ L of TE and lyse by adding 50 μ L of 10% SDS.
4. Add 500 μ L of phenol-chloroform. Mix well, and spin at 12,000*g* for 10 min in a microcentrifuge. Transfer the top (aqueous) layer to a 2.0-mL microcentrifuge tube and add 1 mL of ethanol. Precipitate the DNA at room temperature for 5 min.
5. Spin down the precipitate at 12,000*g* in a microcentrifuge at room temperature. Dry the precipitate.
6. Dissolve the precipitate in 500 μ L of water.
7. Add 50 μ L of 3 *M* sodium acetate and 1 mL of ethanol.
8. Repeat **steps 5** and **6**. Add 2 μ L of RNase A solution, and incubate for 30 min.
9. Repeat **step 7**, centrifuge at 12,000*g*, and dissolve the DNA in 450 μ L of water.
10. Run 5- μ L aliquots on agarose gels, and stain with ethidium bromide. Scan the gel and compare concentrations by densitometry. Adjust the concentrations, and run aliquots again on gels. Repeat until all the samples have identical DNA concentrations (*see Note 3*).

3.5. Preparation of Dot Blots

The layout of the dot blots is shown in **Figs. 2** and **3**.

1. Divide each 400 μ L of DNA sample into two 200- μ L aliquots. To one, add 22 μ L of freshly made 1 *N* NaOH.

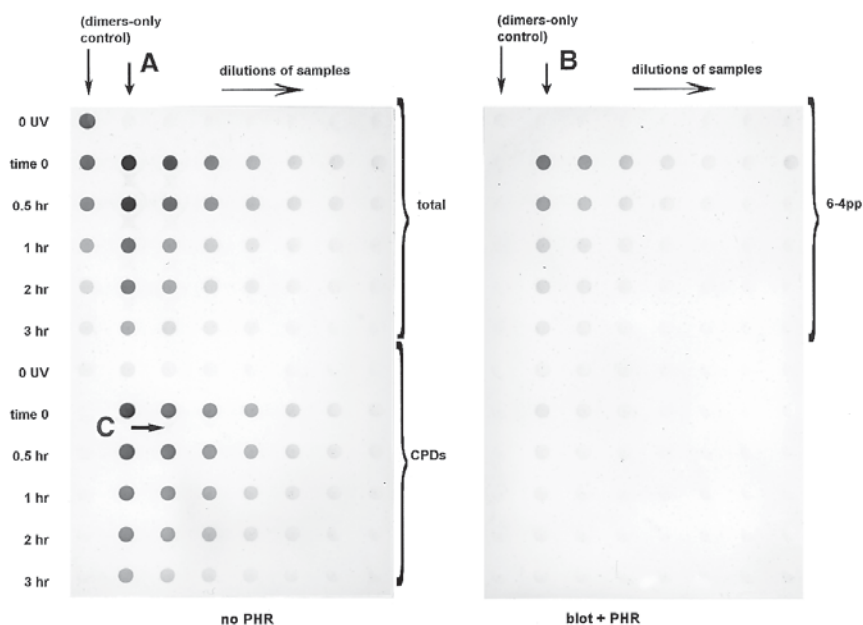


Fig. 3. Dot blots from a yeast repair experiment. Cells were irradiated with 50 J/m^2 and samples were taken immediately and at the postirradiation times indicated. Samples were applied in the array illustrated in Fig. 2. The blot on the right was incubated in photolyase under visible light illumination to photoreactivate CPDs.

2. Incubate at 90°C for 30 min, and cool on ice for 5 min. Then add $110 \mu\text{L}$ of neutralizing solution and $70 \mu\text{L}$ of water.
3. Treat the second $200\text{-}\mu\text{L}$ aliquot the same way, but omit the 90°C incubation.
4. Transfer $100\text{-}\mu\text{L}$ aliquots of all samples into siliconized microtiter plates, and set up a twofold dilution series in 1 M ammonium acetate in a 96-well microtiter plate as indicated in Fig. 2.
5. Transfer samples onto nitrocellulose filters using a vacuum dot-blotting apparatus. Wash the filters in 1 M ammonium acetate and then in 5X SSC, dry, and bake at 80°C .

3.6. Developing the Dot Blots and Quantitating DNA Damage

The method is derived from that described by Wani et al. (8).

1. Incubate the blots overnight in a 1% gelatin solution at 37°C .
2. Incubate the blots destined for measurement of (6-4)PPs in 20 mL of 50 mM Tris-HCl (pH 7.6) containing $100 \mu\text{L}$ of crude photoreactivating enzyme. Incubate the blots in individual plastic boxes for 5 min in the dark followed by 1 h under two 60-W desk lamps, using a piece of plate glass to cut out wavelengths below 320 nm .

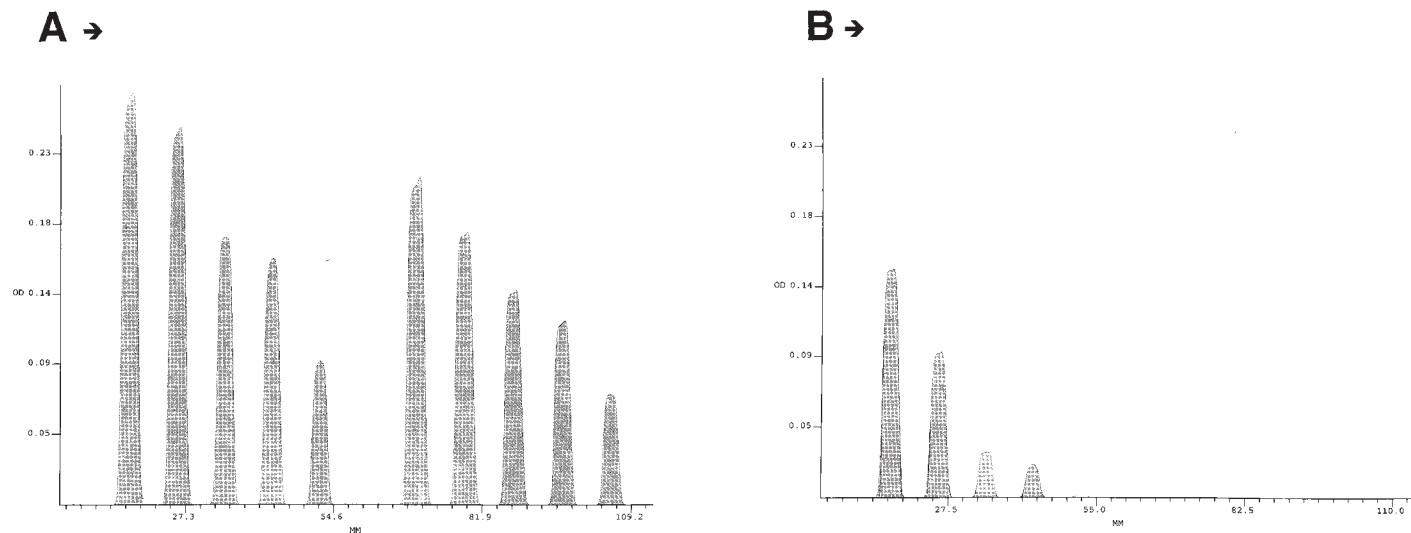


Fig. 4. Scans of tracks labeled **A** and **B** in the blot shown in **Fig. 3**.

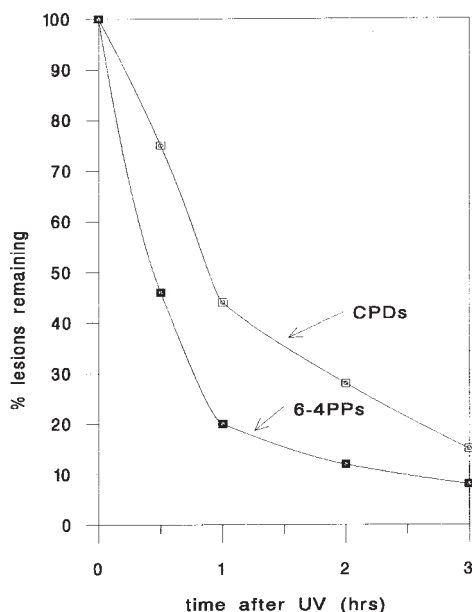


Fig. 5. Repair curves for CPDs and (6-4)PPs calculated from scans of the blot in Fig. 3.

3. Rinse all blots in PBS.
4. Incubate all blots at 37°C for 1 h in 20 mL of PBNT containing 1 mL of denatured crude unirradiated yeast carrier DNA (to bind any nonspecific antibody) and 1 μ L/mL (i.e., 1:1000) anti-UV-DNA polyclonal antiserum.
5. Wash the blots four times in PBX.
6. Incubate the blots for 1 h at 37°C in PBNT containing 1:1000 biotinylated antirabbit antiserum.
7. Wash the blots three times in PBX followed by two washes in TBS.
8. Incubate for 1 h at 37°C in 20 mL of TBS containing 1:1000 alkaline phosphatase-conjugated ExtrAvidin.
9. Wash the blots thoroughly in several changes of TBS, and then incubate, in the dark, in 15 mL of substrate solution for 5–10 min. Watch the reaction and stop before the background begins to go blue, by adding 25 mL of PBS-EDTA. Rinse the blots in water. (See Note 4). Examples of processed dot blots are shown in Fig. 3.
10. Dry the blots and scan using a scanning densitometer with an image analysis facility (Fig. 4). Measure the intensity of the blue color in the dots and set up a calibration curve for each set of samples using the serial dilutions of the time-zero sample as standards (e.g., track C in Fig. 3). Calculate the lesions remaining in the samples from each of the time points as a percentage of the lesions in the time-zero sample (Fig. 5). (See Note 5.)

4. Notes

1. Several types of membrane have been tried for this method. Nitrocellulose gives the lowest background and cleanest results. Nylon gives very high background and is not suitable.
2. Several different enzyme-linked assays and different substrates were used when setting up this assay. The one described here gave a low background and good sensitivity.
3. It is crucial to equalize the DNA in the samples from the various time-points. This cannot be done accurately with a spectrophotometer and is difficult to do accurately even with a fluorimeter. The gel method described is the only one we have found to be adequate.
4. When incubating with the substrate, it is essential to keep the solution dark, to agitate the solution, to keep the blot well covered, and to stop the reaction before the background begins to go blue.
5. Although the method is only semiquantitative, it gives very reproducible results provided care is taken to choose dilutions where the intensity of the blue color is not near saturation, i.e., choose the linear part of the calibration curve.

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Measurement of UV Radiation-Induced DNA Damage Using Specific Antibodies

Ann E. Stapleton

1. Introduction

Measurement of DNA damage can be difficult if the levels of damage are small. For example, the ultraviolet (UV) radiation in sunlight creates cyclobutane pyrimidine dimers (CPDs), but this type of damage is rapidly repaired. The steady-state level of CPDs is thus low, and sensitive methods are required to measure such low levels of UV-induced DNA damage accurately. Antibody–antigen reactions are well understood, and antibody binding can be measured even with very small quantities of antigen. If an antibody that recognizes DNA damage is available, either small or large damage levels can be measured using materials and equipment that are commonly available in molecular biology laboratories.

Monoclonal antibodies (MAbs) specific to CPDs and to a second type of DNA damage, pyrimidine(6,4)pyrimidones, are available from Toshio Mori (*1*). A variety of detection methods can be employed to measure antigen binding; choice of method depends on the sensitivity required and the equipment available. We use ^{35}S -labeled secondary antibody in the method described below to measure CPDs in maize seedlings exposed to solar UV and to measure damage levels in plants exposed to enhanced UV-B from sunlamps (*2–4*). We also routinely use a horseradish peroxidase-coupled secondary antibody with a chemiluminescent detection system for the measurement of CPD damage induced by solar UV (*5*). In our hands, detection methods employing alkaline phosphatase-conjugated secondary antibodies have unacceptably high background levels; a single background spot too near the sample is sufficient to make it impossible to quantify that sample.

In order to achieve reproducible, accurate measurements of antigen amount, assays must be performed with care and all controls included. Dose–response curves should be prepared from each new tissue assayed, and positive and negative controls included on every blot. The typical assay-to-assay variation is about 20%; thus, it is advisable to include as many samples as possible on the same blot and to have multiple replicates of each sample. For discussion of the difficulties in producing good dose–response curves in thick tissues, *see* refs. (6–8).

2. Materials

Molecular biology-grade reagents and sterile distilled water should be used to prepare solutions.

2.1. Preparation of DNA Standards

1. Purified plasmid DNA (e.g., pBluescript, Stratagene, La Jolla, CA).
2. Restriction enzyme and buffer.
3. Germicidal UV-C bulbs, meter, and controller to deliver a known dose of UV-C radiation.
4. Hoefer DynaQuant fluorometer.
5. T4 Endonuclease V (Epicentre, Madison, WI).
6. Agarose.
7. 50 mM NaCl, 4 mM EDTA.
8. Running buffer: 30 mM NaOH, 2 mM EDTA.
9. Loading buffer: 50% glycerol, 1 M NaOH, 0.05% bromocresol green.
10. 0.1 M Tris-HCl, pH 7.5.
11. Ethidium bromide (1 µg/mL).
12. Polaroid camera and Polaroid Type 55 negative film.
13. Densitometer.

2.2. Preparation of Genomic DNA

1. Falcon 2056 tubes or equivalent.
2. Liquid nitrogen.
3. Heated (37°C) shaker that will accommodate the Falcon tubes.
4. Final lysis buffer: 350 mM NaCl, 10 mM Tris-HCl, pH 7.6, 50 mM EDTA, 7 M urea, 2% sarkosyl.
5. Phenol:chloroform (1:1 mixture) made from phenol equilibrated with 10 mM Tris-HCl, pH 7.5.
6. Microcentrifuge tubes.
7. 3 M sodium acetate, pH 5.2.
8. Isopropanol.
9. Ethanol, 70%.

2.3. Binding of DNA to Membrane

1. Nylon membrane (e.g., Hybond-N, Amersham, Chicago, IL). Use a nylon membrane that retains DNA, not a membrane that retains protein.
2. Slot-blot apparatus.

2.4. DNA Damage Detection

1. Phosphate-buffered saline (PBS): 46 g of Na_2HPO_4 , 11.84 g of NaH_2PO_4 , 23.3 g of NaCl, distilled water to 4 L.
2. PBS-T: 0.05% Tween-20 in PBS.
3. Block: 5% nonfat dry milk in PBS-T.
4. Primary anti-CPD MAbs (TDM-2) from Dr. Toshio Mori (*see Note 1*).
5. Seal-a-meal bags and sealer.
6. Fresh ^{35}S -labeled antimouse secondary antibody (Amersham).
7. PBS/1% BSA: 2 g of bovine serum albumin (BSA; protease-free) made up to 200 mL with PBS.
8. PBS/0.1% Tween: 0.2 mL of Tween-20 into 200 mL of PBS.
9. Phosphorimager or X-ray film and densitometer.

3. Methods

3.1. Preparation of DNA Standards

Linear plasmid DNA containing a known number of CPDs is used to standardize the assay.

1. Using the manufacturer's recommended conditions, digest 20 μg of pBluescript or a similar double-stranded plasmid with a restriction enzyme that cleaves the plasmid once and that can be inactivated by heat treatment. Heat-treat the digestion to inactivate the enzyme.
2. Irradiate the plasmid with germicidal UV-C bulbs (*see Note 2*). Divide the sample into three aliquots, in clean weigh boats or empty Petri dish tops. Leave one aliquot unirradiated; use doses of 5 and 10 J/m^2 on the other two aliquots. Dilute the DNA to a concentration of 10 $\text{ng}/\mu\text{L}$. Check the concentration of the standard using a Hoefer DynaQuant according to the manufacturer's instructions (Hoefer, Amersham Pharmacia Biotech, Piscataway, NJ).
3. Treat a portion (~100 ng) of each of the three samples with T4 Endonuclease (TEV) according to the manufacturer's recommendations (*see Note 3*).
4. Run the treated and control (no-TEV) samples on an alkaline agarose as follows (*see Note 4*):
 - a. Prepare a 1.5% alkaline gel by dissolving agarose in 50 mM NaCl and 4 mM EDTA and microwaving. Pour the gel.
 - b. After the gel has solidified, soak it in running buffer for at least 2 h.
 - c. Add 1 vol of loading buffer to the DNA sample, incubate for 15 min at room temperature, and then load the samples.
 - d. Run the gel at 40 V for 3–4 h.

5. Neutralize the gel by soaking it in 500 mL of 0.1 M Tris-HCl, pH 7.5, at room temperature.
6. Stain the gel with ethidium bromide solution, and then destain in water for 30 min.
7. Photograph the gel using Type 55 Polaroid film; develop the negative (*see Note 5*).
8. Quantitate the density of silver grains in the image of the plasmid band on the negative using a densitometer (*see Note 6*).
9. Calculate the number of CPDs in the plasmid DNA in the gel. First, calculate the number of molecules of plasmid found in the nanogram of plasmid in each lane in the gel. Then multiply the number of molecules in the gel by $-\ln P_0$, where P_0 is the scan density of the plasmid band in the TEV-treated sample divided by the scan density of the band in the untreated sample. This will give the number of enzyme-sensitive sites (ESS) in the DNA in the gel (*see Note 7*). For the purposes of this assay, the number of ESS will be assumed to be the same as the number of CPDs.
10. Store the plasmid standards in small aliquots at -80°C .

3.2. Preparation of Genomic DNA

This protocol is a variation on the one described in ref. (9) (*see Note 8*).

1. Harvest ~0.1 g (fresh wt) of *Zea mays* tissue in a Falcon 2056 snap-cap tube, freeze in liquid nitrogen, and store at -80°C .
2. Grind the tissue with a pestle on dry ice with liquid nitrogen: pour liquid nitrogen over the tissue in the tube, let it evaporate, and grind the tissue to a powder (the more ground up the better). Do not allow the tissue to thaw.
3. Add 500 μL of final lysis buffer, and shake in a 37°C shaker/incubator for 10 min.
4. Add 500 μL of phenol:chloroform. Vortex for 10 s. Shake at 37°C for 10 min.
5. Transfer to a microcentrifuge tube, and spin at 12,000g in a microcentrifuge for 5 min. Transfer the supernatant to a new tube.
6. Add 1/10 vol of 3 M sodium acetate, pH 5.2, and an equal volume of isopropanol to the supernatant. Invert to mix. Spin the tube in the microcentrifuge for 2 min.
7. Wash the pellet with 70% ethanol, air-dry, and resuspend the DNA in ~100 μL of TE.
8. Heat to 65°C for 5 min, and vortex repeatedly to get the DNA into solution.
9. Measure the concentration of genomic DNA using a Hoefer DynaQuant according to the manufacturer's instructions (*see Note 9*). **Precise measurement of DNA concentration is critical to the accuracy of this assay.**

3.3. Binding of DNA to Membrane

Use a slot-blotter to fix the DNA to the membrane. Follow the manufacturer's recommendations for denaturation, neutralization, and blotting of the sample. **Bake** the blot according to the manufacturer's instructions to fix the DNA to the membrane permanently. **Do not crosslink the DNA to the blot with UV radiation!**

3.4. DNA Damage Detection

Kits for chemiluminescent detection of HRP may be used; follow the manufacturer's recommendations for blocking, reaction of primary antibody, reaction of secondary antibody, and washes. The protocol for using ^{35}S -labeled anti-mouse antibody is given below. This protocol is from ref. (5) and is reproduced with permission. All the steps should be carried out at room temperature, with agitation by placing the blot in a clean plastic container on a rotating or rocking platform. For all washes, use about 50 mL of solution/wash.

1. Prepare the blocking solution. Block the blot for 1 h.
2. Prepare the PBS-T wash solution. Wash the block with two quick rinses, followed by one 15-min rinse and two 5-min rinses.
3. Dilute the primary antibody 1:2000 in PBS; 75 $\mu\text{L}/\text{cm}^2$ of membrane will be needed. Place the blot in a seal-a-meal bag, and seal all sides. Cut one corner open, and pour in the diluted primary antibody. A small clean funnel is helpful. Squeeze out any air bubbles, and seal the bag. Incubate for 1 h with agitation.
4. Cut open a corner of the bag and remove the primary antibody (*see Note 10*). Place the blot in a clean plastic container and wash with PBS-T, using two quick rinses, one 15-min rinse, and two 5-min rinses.
5. Dilute the secondary antibody 1:1000 into PBS/1% BSA; 75 $\mu\text{L}/\text{cm}^2$ of membrane will be needed. Observe radioactivity precautions. Place the blot in a seal-a-meal bag, and seal all sides. Cut one corner open, and pour in the diluted secondary antibody. Squeeze out any air bubbles (watch for release of radioactivity), and seal the bag. Incubate for 1 h with agitation.
6. Cut open a corner of the bag, and remove the secondary antibody (*see Note 11*). Wash the blot once with PBS/1% BSA for 15 min.
7. Wash the blot three times for 10 min each time with PBS/0.1% Tween. Allow the blot to air-dry.

3.5. Signal Quantitation (*see Notes 12 and 13*)

1. If a phosphorimager is available, use it according to the manufacturer's instructions for exposure of the blots and quantitation of the signal.
2. If no imager is available, blots may be exposed to X-ray film, developed, and the signal quantified by densitometry.

4. Notes

1. Dr. Toshio Mori's fax number is 81-7442-5-7657. Anti-CPD MABs are also available commercially from Kamiya Biomedical Company (Thousand Oaks, CA), but these have not been tested in the protocol described here.
2. A UV crosslinker may be used for the UV-C irradiations.
3. As a precaution, also treat unirradiated plasmid DNA with TEV to test for the presence of nonspecific endonuclease activity in the enzyme preparation.

4. This protocol was adapted from ref. (10). Methods for alkaline agarose-gel electrophoresis can also be found in ref. (11) and Chapters 16, 18, and 32.
5. Several exposures may be required in order to ensure that the exposure is in the linear range of the film. The band intensity of the no-TEV sample can also be used to confirm that all the samples are at the same concentration.
6. A flatbed scanner and NIH Image 1.4 or other commercially available scanners and software will work also.
7. A more extensive explanation of this measurement method may be found in ref. (12). See also Chapters 15 and 21.
8. Most DNA preparation methods will produce genomic DNA that will work in this assay. It is critical, however, to remove RNA (which can also contain dimers) and, if chemiluminescent detection is used, to make sure there is no dark-colored material left in the preparation that will interfere with emission of light. You can never extract all the DNA, and any DNA that is crosslinked to protein or cell-wall material will be lost.
9. If a DynaQuant is not available, agarose-gel electrophoresis with known standards can be used to measure the concentration of genomic DNA. Ensure that the genomic DNA runs as a tight band in a 1.2% agarose gel after electrophoresis for only a short distance. Photograph the gel with Polaroid Type 55 film, develop the negative according to the manufacturer's directions, and quantitate the amount of DNA compared to standards of known concentration by densitometry of the negative.
10. Diluted antibody may be stored at 4°C and reused twice within 2 wk; discard when a precipitate forms.
11. Diluted antibody may be stored at 4°C and reused within 2 wk; discard into radioactive waste when a precipitate forms.
12. The large linear exposure range of phosphorimagers makes quantitation of the signal significantly easier. If film is used, several exposures may be required in order to ensure that all samples and standards are within the linear exposure range of the film.
13. If there is any substantial sample-to-sample variation in the amount of DNA on the blot, the accuracy of the assay can be compromised. If CPD levels are low enough not to interfere with hybridization, it is possible to check for such variation by removal of the antibody and hybridization of the blot to a probe made from genomic DNA (11).

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Quantification of Photoproducts in Mammalian Cell DNA Using Radioimmunoassay

David L. Mitchell

1. Introduction

Radioimmunoassay (RIA) is a competitive binding assay between an unlabeled and a radiolabeled antigen for binding to antibody raised against that antigen. For the development of this technique, Yalow and Berson (*1*) received the Nobel Prize in Medicine. For detailed theory and troubleshooting of RIA, *see* Harlow and Lane (*2*) and Chard (*3*). We have adapted this technique to the measurement of specific DNA photoproducts in the DNA of UV-irradiated cells (*4,5*). The following description is given for quantification of cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6-4)pyrimidinone photoproducts ([6-4]PPs) in DNA using RIA. For convenience, the radiolabeled antigen is referred to as the “probe,” and the unlabeled competitor as the “sample” or “standard.” The amount of radiolabeled antigen bound to antibody is determined by separating the antigen–antibody complex from free antigen by secondary antibody (**Fig. 1**). The amount of radioactivity in the antigen–antibody complex in the presence of known amounts of competitor (i.e., standards) can then be used to quantify the amount of unknown sample present in the reaction. The sensitivity of the RIA is determined by the affinity of the antibody and specific activity of the radiolabeled antigen. Using high-affinity antibody and probe labeled to a high specific activity, the reaction can be limited to such an extent that extremely low levels of damage in sample DNA can be detected. This particular procedure has resulted from 15–20 years of research and has proven to be a reliable and facile technique for measuring DNA damage and repair end points. That is not to say that modifications of this basic procedure will not be as productive or useful in DNA damage and repair studies.

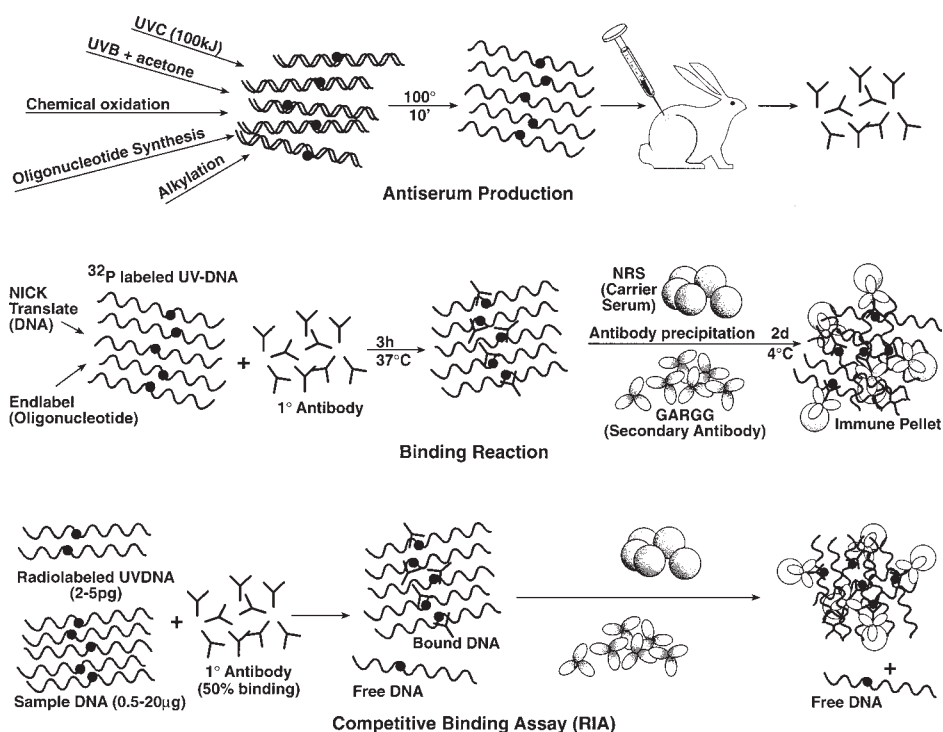


Fig. 1. Diagram of RIA protocol. Top, Antibodies are raised against a specific type of DNA lesion, e.g., CPD. A variety of methods and treatments can be used to damage or modify bases in DNA for use as an immunogen. Middle: Binding activity of antisera is characterized. Bottom, RIA is used to measure lesion levels in sample DNA.

2. Materials

2.1. Preparation of Immunogen

1. H₂O (HPLC- or Millipore-filtered, Millipore Corp., Bedford, MA).
2. Salmon testes or calf thymus DNA (Sigma, Rochester, NY). (*See Note 1.*)
3. Acetone.
4. UV-B source: The UV-B source consists of four Westinghouse FS20 sunlamps filtered through cellulose acetate (Kodacel from Kodak, St. Louis, MO) with a wavelength cutoff of 290 nm (6). Dosimetry is determined with an appropriate photometer/radiometer (e.g., IL1400 photometer coupled to a SCS 280 probe, International Light, Newburyport, MA).
5. UV-C source: The UV-C source consists of a bank of 5 Philips Sterilamp G8T5 bulbs emitting predominately 254 nm of light.
6. Methylated bovine serum albumin (MBSA) (*see Note 2.*)

2.2. RIA

1. Polyd(A):polyd(T) or *Clostridium perfringens* DNA (see **Note 3**).
2. DNA nick-translation kit (Boehringer Mannheim, Indianapolis, IN).
3. NICK column (Amersham-Pharmacia Biotech, Inc., Piscataway, NJ).
4. ^{32}P -Labeled deoxynucleotide triphosphates (dNTPs).
5. 12-mm Culture tubes (Fisher, Pittsburgh, PA or VWR Scientific Products, Chester, PA.) (see **Note 4**).
6. RIA buffer: 1X TES + 0.2% gelatin (type B: bovine skin; Sigma). (See **Note 5**.)
7. Normal rabbit serum (Calbiochem Corp., San Diego, CA). Store frozen in 200- μL aliquots. (See **Note 6**.)
8. Goat antirabbit IgG (Calbiochem). Store frozen in 0.5-mL aliquots (See **Note 7**.)
9. Tissue solubilizer (NCS-II from Amersham) supplemented with 10% (v/v) H_2O .
10. Scintillation cocktail (e.g., ScintiSafe from Fisher) supplemented with 1 mL/L acetic acid to eliminate chemoluminescence generated by the tissue solubilizer.

2.3. Cell Culture and DNA Isolation

1. ^{14}C -labeled thymidine deoxyribonucleoside (^{14}C -TdR).
2. 10X TES: 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1.5 M NaCl.
3. Lysis buffer A: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% sodium dodecyl-sulfate (SDS), and 0.3 mg/mL proteinase K (Boehringer Mannheim) (see **Note 8**).
4. Lysis buffer B: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% SDS, 100 $\mu\text{g}/\text{mL}$ DNase-free RNase A (Boehringer Mannheim) (see **Note 9**).
5. Sevag: chloroform:isoamyl alcohol; 24:1.
6. 5 M Sodium acetate (see **Note 10**).
7. Ethanol, 100%, 70%.
8. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
9. 15-mL Polypropylene centrifuge tubes.
10. 30-mL Corex tubes.

3. Methods

3.1. Preparation of Immunogen

1. Dilute salmon testes or calf thymus DNA to 1 mg/mL in 10 mL of sterile H_2O (as determined by optical density at 260 nm).
2. UV-irradiate the diluted double-stranded DNA using one of the following protocols:
 - a. Prepare the immunogen for anti-CPD sera by irradiating DNA diluted in 10% acetone (final concentration, v/v) (7) with $\sim 75 \text{ kJ}/\text{m}^2$ UV-B light (see **Note 11**) in a glass 100-mm plate.
 - b. Prepare the immunogen for anti-(6-4)PP sera by irradiating DNA with $60 \text{ kJ}/\text{m}^2$ UV-C light (see **Note 12**).
3. Heat-denature the UV-irradiated DNA at 100°C for 10 min.
4. Electrostatically couple the single-stranded UV-irradiated DNA to MBSA (see **Note 13**) (8).

3.2. Immunization Schedule

1. Initially, inject subcutaneously 4 New Zealand White female rabbits (*see Note 14*) at 10 sites (100 μ L each) with 0.5 mL of immunogen mixed with an equal volume of Freund's Complete Adjuvant (final concentration of UV-DNA is 0.1 mg/mL).
2. Subsequently, inject rabbits using the same protocol at 2-wk intervals, except mix Freund's Incomplete, rather than Complete, Adjuvant with 0.5 mL of immunogen.
3. At 10–12 days following the second injection, draw 1 mL of serum and evaluate the binding affinity using immunoprecipitation (*see Subheading 3.3.*).
4. Continue immunization at 2-wk intervals until sufficient binding activity is attained, at which time draw antisera (60–80 mL) from the animal using heart puncture.
5. Dispense the antisera into 1-mL aliquots and store at -20°C (*see Note 15*).

3.3. Determination of Antiserum Binding Using Immunoprecipitation (Fig. 1)

1. Nick-translate DNA (0.1 μ g) with ^{32}P -dCTP and/or ^{32}P -TTP to give a specific activity of $\sim 5 \times 10^8$ – 10^9 cpm/ μ g. A typical reaction includes:
 - a. 2 μ L of 10X nick translation buffer.
 - b. 2 μ L dATP (for poly[dA]:poly[dT]); or 2 μ L each dATP and dGTP (for DNA).
 - c. 0.5 μ L of poly(dA):poly(dT) or *C. perfringens* DNA (diluted to 20 μ g/100 μ L).
 - d. 12.5 μ L of ^{32}P -TTP at 10 mCi/mL.
 - e. 3–4 μ L of DNase I/DNA polymerase I enzyme mix (from kit).
 - f. Incubate at 15°C for 30–45 min.
 - g. Separate radiolabeled ligand from free dNTPs using a Nick column equilibrated with 1X TE buffer. Elute with TE.
2. Irradiate the ^{32}P -labeled probe with 30 kJ/m² UV-C light (*see Notes 12 and 16*).
3. Restore the volume (owing to evaporation) with H₂O and dilute 2500- to 5000-fold in RIA buffer (yielding 2.5–5.0 pg of probe in 50 μ L of buffer) (*see Note 17*).
4. Add 1 mL of RIA buffer to duplicate 12-mm disposable culture tubes.
5. Add 50 μ L of antiserum diluted in RIA buffer at half-log increments from 1:1000 to 1:1,000,000 (dilution prior to dispensing). Dispense duplicate tubes without antiserum to determine background levels.
6. Add 50 μ L of diluted ^{32}P -labeled probe (from **step 3**), and vortex well.
7. Incubate 3–4 h with gentle rotation (optional) in a 37°C dry incubator.
8. Separately add 50 μ L of normal rabbit serum diluted 1:40 in RIA buffer and 50 μ L of goat antirabbit IgG diluted 1:20, and vortex well.
9. Incubate at 4°C for 2 d until the immune pellet (translucence) develops.
10. Centrifuge the tubes at $\sim 2500g$ for 30–45 min at 10°C .
11. Decant the supernatant, invert the tubes onto absorbant paper in a test tube rack, and drain for 5–10 min (*see Note 18*).
12. Swab the lip of the test tube with a cotton-tipped applicator wrapped in tissue (to remove any accumulated liquid).

13. Add 100 μL of NCS tissue solubilizer supplemented with 10% H_2O , and incubate at 37°C (or room temperature) with rotation until the immune pellet is completely dissolved (*see Note 19*).
14. Add 2 mL of scintillation cocktail supplemented with 1 mL/L acetic acid, and vortex.
15. Decant the sample into a 20-mL scintillation vial, and wash twice with 4 mL of additional scintillation cocktail.
16. Count ^{32}P using a liquid scintillation counter.

3.4. Treatment and Isolation of Cultured Mammalian Cell DNA

1. Plate $2.5\text{--}3 \times 10^6$ cells in 7×100 mm plates (duplicate or triplicate plates can be used) with medium (e.g., α -MEM) containing 0.005–0.01 mCi/mL ^{14}C -TdR 2 d prior to irradiation (*see Note 20*).
2. For a DNA repair experiment, irradiate all (but one –UV control) plate with $10\text{--}20 \text{ J/m}^2$ UV-C light or UV-B equivalent (*see Note 21*). The perimeter of the plates should be swabbed with a cotton-tipped applicator to remove cells that would otherwise be shielded from the radiation. Pour off the medium, and wash the plates once with 1X TES. Harvest the unirradiated sample as a control.
3. Harvest one irradiated plate (with duplicate) at the time of irradiation by scraping the cells with a rubber policeman into a 15-mL polypropylene centrifuge tube. (Trypsinization can also be used to lift adherent cells from the plate.) Additional plates should be harvested at 1.5, 3, 6, 24, and 48 h (for example) postirradiation for repair studies.
4. Centrifuge at $\sim 150g$ for 5 min to pellet the cells; decant the buffer.
5. Add 4 mL of lysis buffer A or B (*see Notes 8 and 9*), mix vigorously, and incubate overnight at 37°C or for 2–3 h at 60°C .
6. Extract with 4 mL of Sevag, and transfer the aqueous phase to a 30-mL Corex tube.
7. Add 0.4 vol (1.6 mL) of 5 M sodium acetate and 2.5 vol (14 mL) of ice-cold absolute ethanol. Place in a freezer overnight.
8. Centrifuge the sample at 10,000 rpm in a SS-34 rotor (12,000g) at 0°C for 20 min to pellet the DNA. Decant the supernatant away from the side containing the pellet.
9. Wash the pellet with 5–10 mL of ice-cold 70% ethanol.
10. Allow the tube to dry inverted for 30–60 min at room temperature (not to complete dryness) and resuspend the pellet in 1.5 mL of sterile H_2O or TE buffer. Allow several hours with periodic vortexing for the pellet to resuspend completely.
11. Determine the DNA concentration using absorption or spectrofluorometry (*see Note 22*).
12. After heat denaturation, count 20–50 μL to determine the level of ^{14}C -DNA (if applicable).
13. Place in refrigerator at 4°C for short-term or in -20°C freezer for long-term storage.

3.5. Competitive Binding Assay (RIA)

The RIA is simply the basic immunoprecipitation reaction outlined in **Subheading 3.3.** into which a standard or sample DNA has been added to compete

with the radiolabeled probe for antibody binding (**Fig. 1**). Therefore, the procedure is exactly the same as that used for immunoprecipitation with the following additions/modifications:

1. A single dilution of antiserum is used. This dilution is determined from immunoprecipitation analyses of binding activity (**Subheading 3.3.**) and should yield 30–60% of the radiolabeled probe in the immune pellet.
2. For quantification of CPDs or (6-4)PPs, a dose–response of heat-denatured UV-irradiated salmon testes DNA is used as standard (e.g., **Fig. 2A**). We routinely use doses of 3, 10, 30, 100, and 300 J/m² as our standard curve, and assay the same amount of standard as sample DNA. When relative, rather than exact, amounts of CPDs or (6-4)PPs are adequate for experimental purposes (as in DNA repair experiments), the sample harvested at the time of irradiation is titrated in half-log increments to determine the optimal amount required for assay (**Fig. 2B**).
3. Unlabeled competitor mammalian DNA, radioactive ligand, and diluted antibody are incubated together for 3 h at 37°C with gentle rotation (optional). As above, it is prudent to perform a preliminary titration of sample DNA to determine the amount required for adequate inhibition in the RIA. The total volume of sample DNA added can vary within certain limitations (see **Note 23**).

3.6. Data Analysis

1. Sample Excel spreadsheets are shown in **Fig. 2**. Formulae for quantifying CPDs are shown in **Fig. 2A**. An identical spreadsheet can be used to quantify (6-4)PPs.
2. A sample Excel spreadsheet for quantification of relative photoproducts (PDs) remaining at specific times post-UV irradiation (e.g., in a DNA repair experiment) is shown in **Fig. 2B**.

4. Notes

1. Commercial DNA does not usually require repurification. However, the purity should be checked using the $A_{260/280}$ with values >1.7 acceptable.
2. MBSA can be frozen and thawed *ad infinitum*.
3. Both CPD and (6-4)PP frequencies are greatest in nucleic acid substrates containing a high A + T:G + C ratio. Therefore, optimal substrates for the radiolabeled probe include *C. perfringens* DNA as well as the homopolymer poly(dA):poly(dT).
4. We use 12-mm culture tubes that have colored labels. This helps separate the components of the RIA (i.e., binding conditions, standard curve, sample groups) for more facile visual recognition and less pipeting error.
5. RIA buffer consists of 1X TES to which 0.2% gelatin (w/v) (Sigma) has been added to reduce nonspecific binding. The gelatin is heated into solution using a hot plate magnetic stirrer (not a microwave) and heated to precisely 39–40°C. Overheating (by as much as 1°C) will result in prohibitive background! The cause of this is unknown.

A

Tube #	Sample	UV Dose	ul/(x)ug	ug/ml	cpm	cpm-bkg	%inhibition	CPD/mb		
1	-Ab									
=A2+1	+Ab					=AVERAGE(F2,F3) =F4-\$G\$3 =F5-\$G\$3	=AVERAGE(G4,G5)			
=A4+1	standard	3	=(x/E6)*1000	x		=F6-\$G\$3 =F7-\$G\$3	=(1-(\$G6/\$H\$5))*100 =(1-(\$G7/\$H\$5))*100	=LOG(C6*8.1) =LOG(C7*8.1)	=RSQ(H1:H5,I1:I5)	Corr.Coef.
=A6+1	standard	10	=(x/E8)*1000	x		=F8-\$G\$3 =F9-\$G\$3	=(1-(\$G8/\$H\$5))*100 =(1-(\$G9/\$H\$5))*100	=LOG(C8*8.1) =LOG(C9*8.1)	=SLOPE(H1:H5,I1:I5)	Slope
=A8+1	standard	30	=(x/E10)*1000	x		=F10-\$G\$3 =F11-\$G\$3	=(1-(\$G10/\$H\$5))*100 =(1-(\$G11/\$H\$5))*100	=LOG(C10*8.1) =LOG(C11*8.1)	=INTERCEPT(H1:H5,I1:I5)	Y-intercept
=A10+1	standard	100	=(x/E12)*1000	x		=F12-\$G\$3 =F13-\$G\$3	=(1-(\$G12/\$H\$5))*100 =(1-(\$G13/\$H\$5))*100	=LOG(C12*8.1) =LOG(C13*8.1)		
=A12+1	sample	x	=(x/E14)*1000	x		=F14-\$G\$3 =F15-\$G\$3	=(1-(\$G14/\$H\$5))*100 =(1-(\$G15/\$H\$5))*100	=10^(((\$H14-\$K\$10)/\$K\$8) =10^(((\$H15-\$K\$10)/\$K\$8)		
=A14+1	sample	y	=(x/E16)*1000	x		=F16-\$G\$3 =F17-\$G\$3	=(1-(\$G16/\$H\$5))*100 =(1-(\$G17/\$H\$5))*100	=10^(((\$H16-\$K\$10)/\$K\$8) =10^(((\$H17-\$K\$10)/\$K\$8)		

B

Tube #					cpm	cpm-bkg	%inhibition			
1	-Ab									
=A2+1	+Ab					=AVERAGE(G2,G3) =G4-\$H\$3 =G5-\$H\$3	=AVERAGE(H4,H5)			
	Cell line	Dose	Time (h)	ul/(x)cpm	cpm/20 ul			% PD remaining		
=A4+1	-UV	0h		(y)ul		=G7-\$H\$3 =G8-\$H\$3	=AVERAGE(H7,H8)			
=A7+1	+UV	0h		=E13*0.1		=G9-\$H\$3 =G10-\$H\$3	=(1-(\$H9/#REF!))*100 =(1-(\$H10/#REF!))*100	=LOG(J9) =LOG(J10)	=RSQ(I9:I14,K9:K14)	Corr Coef.
=A9+1	+UV	0h		=E13*0.3		=G11-\$H\$3 =G12-\$H\$3	=(1-(\$H11/#REF!))*100 =(1-(\$H12/#REF!))*100	=LOG(J11) =LOG(J12)	=SLOPE(I9:I14,K9:K14)	Slope
=A11+1	+UV	0h		(y)ul		=G13-\$H\$3 =G14-\$H\$3	=(1-(\$H13/#REF!))*100 =(1-(\$H14/#REF!))*100	=LOG(J13) =LOG(J14)	=INTERCEPT(I9:I14,K9:K14)	Y-intercept
=A13+1	+UV	1.5		(y)ul		=G15-\$H\$3 =G16-\$H\$3	=(1-(\$H15/#REF!))*100 =(1-(\$H16/#REF!))*100	=10^(((\$I15-\$L\$13)/\$L\$11) =10^(((\$I16-\$L\$13)/\$L\$11)		
=A15+1	+UV	3		(y)ul		=G17-\$H\$3 =G18-\$H\$3	=(1-(\$H17/#REF!))*100 =(1-(\$H18/#REF!))*100	=10^(((\$I17-\$L\$13)/\$L\$11) =10^(((\$I18-\$L\$13)/\$L\$11)		
=A17+1	+UV	6		(y)ul		=G19-\$H\$3 =G20-\$H\$3	=(1-(\$H19/#REF!))*100 =(1-(\$H20/#REF!))*100	=10^(((\$I19-\$L\$13)/\$L\$11) =10^(((\$I20-\$L\$13)/\$L\$11)		
=A19+1	+UV	24		(y)ul		=G21-\$H\$3 =G22-\$H\$3	=(1-(\$H21/#REF!))*100 =(1-(\$H22/#REF!))*100	=10^(((\$I21-\$L\$13)/\$L\$11) =10^(((\$I22-\$L\$13)/\$L\$11)		

Fig. 2. Microsoft Excel spreadsheets showing calculations used in RIA experiments. (A) Formulae used for quantifying CPDs. (B) Formulae for quantifying relative levels of photoproducts (PD).

6. Normal rabbit serum (NRS) from Calbiochem has been titrated, and we have found that a 1:40 dilution is optimal for immune pellet formation. Obviously other sources are readily available. However, we suggest that titrations be performed in the context of the binding assay to determine the optimal dilution.
7. Goat antirabbit IgG can be purchased from Calbiochem in bulk or smaller aliquots. The bulk product requires a greater concentration than the individual 5-mL aliquots, and we suggest, as above, that the optimal dilution be determined using the immunoprecipitation protocol.
8. Lysis buffer A is used for "crude" extractions in which the DNA has been prelabeled with ^{14}C -TdR and the RIA is set up to determine relative amounts of photoproduct remaining in a DNA repair experiment (as shown in **Fig. 2B**). In such an experiment, actual quantification of damage is not required, since the 0 h sample is titrated to serve as a standard curve. In this case, it is only necessary to analyze equivalent amounts of ^{14}C .
9. Lysis buffer B is used when number of photoproducts per megabase of DNA are required. In this case, the concentration of the DNA sample is critical, and care must be taken to assure accurate quantification. A more standard DNA isolation procedure is called for, which includes lysis in the presence of RNase, followed by proteinase K digestion, organic extractions with equal volumes of phenol, phenol:Sevag (1:1), and Sevag, and precipitation with 2 vol of ethanol in the presence of 0.4 vol of 5 M ammonium acetate.
10. 5 M Sodium acetate should be filter-sterilized and stored at 4°C. This buffer should be checked prior to use for growth of contaminating organisms.
11. At a distance of ~10 cm, the fluence rate is ~5 J/m²/s. Therefore, exposure times of ~4 h are required for adequate CPD induction. Dialyze the DNA extensively postirradiation to remove any acetone.
12. At a distance of ~20 cm the fluence rate is ~14 J/m²/s, and at this fluence rate, the average duration of exposure is ~1.2 h.
13. MBSA is added dropwise with a Pasteur pipet (~50 μL /drop) until the UV-DNA is significantly translucent (i.e., until further addition of MBSA does not change the cloudiness of the solution).
14. We have found that individual rabbits have very different immune responses (5). Hence, we recommend that 4 animals be used for raising anti-UV DNA antibodies.
15. Repeated freezing and thawing of antisera is to be strictly avoided, since this can severely reduce binding activity.
16. Facile irradiation of small volumes of DNA can be achieved using a 25-mm plate or 24-well culture plate in which a depression has been made in a parafilm covering. By drilling a small hole in the bottom of the well, air is released to prevent puckering.
17. The amount of probe added to the RIA determines its sensitivity. It is essential to use 10 pg or less, and have enough cpm in the assay to yield useful binding (and inhibition) data. Therefore, if a 5000 dilution of probe leaves <500 cpm in 50 μL , a greater concentration must be used. Good assay conditions should be limited to at least 500 cpm/50 μL (added to reaction) at a probe dilution not to exceed

- 1:1250. The probe is irradiated with a UV-C light source (*see Note 12*); a dose of 30 kJ/m² is approximately a 30- to 40-min exposure with the probe at 20 cm from the bulbs.
18. Pellets can be inverted for 15–30 min to allow drainage, but care should be taken that pellets do not slide down the face of the tube.
 19. It is extremely important that the immune pellet be completely solubilized, but not allowed to dry. Partial solubilization will result in bad duplicates.
 20. The amount of ¹⁴C-TdR added to the cells and the duration of the prelabeling depend on the doubling time of the particular cells being studied. Our experience with transformed human fibroblasts and Chinese hamster ovary cells (with doubling times of 24 h) has shown that 2 d of incubation with label results in specific activities of 1000–3000 cpm/μg DNA.
 21. We have found that unfiltered FS20 (UV-B) sunlamps induce 1/10 the amount of CPDs in DNA as UV-C irradiation. FS20 sunlamps filtered through cellulose acetate induce 1/100 the amount of damage as that produced by UV-C irradiation.
 22. After precipitation and washing (with 70% ethanol), duplex DNA is quantified using absorbance at 260 nm (assuming the A_{260/280} is >1.7) or spectrofluorometry using a DNA-specific dye (e.g., Hoescht or DAPI). From **Fig. 2A**, it is evident that equivalent amounts of standard and sample are required to determine photo-product concentrations.
 23. Sample volumes <100 μL do not significantly affect the reaction conditions (e.g., total binding). Sample volumes >100 μL can be used, however the total reaction volume should be increased accordingly (i.e., doubled).

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Monitoring Removal of Cyclobutane Pyrimidine Dimers in *Arabidopsis*

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1. Introduction

Ultraviolet (UV) radiation that overlaps the absorption spectrum of DNA induces a variety of photoproducts. Because stratospheric ozone screens out shorter UV wavelengths, DNA-damaging solar irradiance at the terrestrial surface is confined to the UV-B band (290–320 nm). However, germicidal lamps with outputs in the UV-C range induce both principal classes of UV-light photoproducts in DNA, cyclobutane pyrimidine dimers (CPDs) and pyrimidine-[6-4']-pyrimidinone photoproducts ([6-4] photoproducts; [6-4]PPs), in about the same proportions—70–80% CPDs, 20–30% (6-4)PPs—as does UV-B radiation, so the former is frequently used in laboratory situations. Other photoproducts account for 1–2% at most of UV photoproducts, so CPDs and (6-4)PPs are undoubtedly responsible for most of the cytotoxicity, mutagenicity, and carcinogenicity of UV light. It seems highly likely that ongoing depletion of stratospheric ozone will significantly increase solar UV-B irradiance of the biosphere during the next decade or two. This has triggered increased interest in the consequences for green plants, which are exposed more or less constantly to sunlight, on which they depend for photosynthetic energy and development signals. A prerequisite to understanding mechanisms by which plants repair and/or tolerate UV-light damage to their DNA is the availability of accurate, sensitive, and relatively simple methods to measure photoproducts. Here we have focused on CPDs, the most prevalent UV photoproducts in DNA, which are known to be cytotoxic and mutagenic.

CPDs were the first photoproducts to receive intensive biological study. Unlike (6-4)PPs, they are stable to acid treatments strong enough to release (dimerized) bases from DNA. Chromatographic analysis of hot-acid hydroly-

sates remains an accurate, highly reproducible technique for measuring larger levels of CPDs in DNA (*see* Chap. 11). However, it is relatively insensitive, and depends on quantitative measurements of pyrimidines across an entire chromatogram, by radioactivity counting or other means, so each determination is quite time-consuming. The availability of specific antibodies against CPDs and against (6-4)PPs has made possible very sensitive assays for detecting these photoproducts in DNA (*see* Chaps. 12–14), but these assays typically yield relatively noisy data, with large standard errors.

CPD-glycosylase/AP-lyase (“UV endonuclease”) enzymes efficiently and specifically cleave the *N*-glycosidic bond linking the 5'-ward dimerized base to deoxyribose and (less efficiently) catalyze strand cleavage, by a base elimination mechanism, of the phosphodiester linkage 3' to the resulting apyrimidinic site. (Treatment with alkali efficiently accomplishes the same strand cleavage.) The number of DNA sites cleaved by these so-called UV endonucleases (sometimes termed endonuclease-sensitive sites [ESS]) is thus a direct and sensitive measure of the number of CPDs. Various methods to determine frequencies of ESS in UV-irradiated DNA have been described.

In the technique extensively developed by Bohr and coworkers (*1*; *see also* Chap. 21), the probability that a given specific DNA fragment is free from CPDs is determined, after digestion with a particular restriction endonuclease, by exhaustive treatment with a CPD-specific UV endonuclease, separation of the single-stranded DNA (ssDNA) products by gel electrophoresis in alkali, and measurement of the DNA in the band corresponding to full-size (therefore no ESS) fragments by quantitative transfer to nitrocellulose paper and hybridization with a specific radiolabeled probe (Southern blotting). The average number of Poisson-distributed ESS (therefore CPDs) follows from the observed probability that any given fragment contains no ESS. The advantages of this technique are that only a single discrete band need be analyzed, and that repair in different specific gene regions, or even specific DNA strands, can be compared. The disadvantages are that not much less than one ESS per resolvable DNA fragment (typically 10–20 kb) can be detected, and that the particular fragments chosen may not be representative of the genome.

When an entire genome is to be analyzed, it is necessary to measure the decrease in the number-average ssDNA molecular weight (mol wt) when an irradiated DNA sample is treated with UV endonuclease. Molecular weight is a measure of the number of ssDNA ends, and therefore of ESS, in the DNA population. Freeman, and coworkers (*2*) have developed and refined techniques for resolving large ssDNA fragments by pulsed-field electrophoresis in agarose gels alongside size standards, followed by characterization of the mol-wt

distributions by staining with ethidium bromide, and quantitative video camera scanning and image analysis (2). Careful attention to extraction of DNA without mechanical breakage has pushed the sensitivity to extraordinary levels—approaching 1 CPD/ 10^9 nucleotides. However, the “one-of-a-kind” nature of the expensive instrumental/analytical package employed, and the resources and expertise needed to put it together, have precluded its widespread use, although a simplified version is presented in Chapter 16. We describe here a “lower-tech” method that is less sensitive, but more user-friendly, sedimentation in alkaline-sucrose gradients. This technique, applicable to any uniformly radio-labeled plant DNA, has proven useful in studies of DNA repair in *Arabidopsis thaliana* (3,4).

A disadvantage of all of the techniques described above is that the damage signal, photoproduct frequency per unit amount of DNA, decreases both when damage is removed and when it is diluted out by semiconservative DNA replication. In the case of rapidly growing mammalian cells, accurate application of the Hanawalt-Bohr technique requires density labeling of DNA products of postirradiation semiconservative replication, and their removal by buoyant-density sedimentation in CsCl (*see* Chap. 21). Where repair is fast, and replication demonstrated to be slow or negligible, as for photoreactivation in mature plant tissues (3,4), dilution by replication may be ignored. However, detection methods based on uniform radiolabeling of DNA *in vivo* do provide a means to circumvent the replication problem (*see Subheading 2.1. below*).

2. Materials

2.1. Radiolabeled Plant DNA: General Considerations

Defined media, such as the standard Murashige-Skoog/sucrose agar used for growth of *Arabidopsis* in Petri dishes (3), provide an opportunity to radiolabel plant DNA. *Arabidopsis* seedlings readily take up and incorporate [^3H] thymidine in agar, perhaps because their very small seeds contain few stored nutrients. Attempts to radiolabel similarly the DNA in wheat, maize, and rice seedlings have been unsuccessful (J. Hays, unpublished results). Deoxyadenosine at concentrations that stimulate [^3H] thymidine incorporation into bacterial DNA (100 $\mu\text{g/mL}$) is toxic to *Arabidopsis* seedlings. Injection of excess unlabeled thymidine into the agarose, after UV irradiation for instance, makes subsequently semiconservatively replicated DNA invisible to radiolabel-based assays (A. B. Britt, personal communication).

A number of methods for extracting DNA from plants have been reported. We describe below one procedure for *in vivo* radiolabeling and isolation of *Arabidopsis* DNA.

2.2. Radiolabeling of *Arabidopsis* DNA In Vivo and Extraction of DNA

1. Murashige-Skoog salt mixture (pH 5.7) (Gibco BRL, Gaithersburg, MD).
2. MSS-agar: 0.43% Murashige-Skoog salt mixture, 1% sucrose, 0.001% nicotinic acid, 0.01% pyridoxine-HCl, 0.004% glycine, 0.0001% thiamine-HCl, 0.01% myoinositol, 0.8% agar (Difco, Detroit, MI).
3. [^3H] Thymidine, added to MSS-agar to about 2 $\mu\text{Ci/mL}$.
4. Small mortar and pestle for grinding; liquid N_2 .
5. Tissue resuspension buffer: 2% (w/v) cetyltrimethylammonium bromide (CTAB), 100 mM Tris-HCl, pH 8.0, 20 mM Na_2EDTA , 1.4 M NaCl, 1% polyvinylpyrrolidone (mol wt 4×10^4).
6. Crude yeast tRNA.
7. Chloroform:isoamyl alcohol (24:1, v/v).
8. CTAB salt solution: 10% CTAB (w/v) in 0.7 M NaCl.
9. CTAB precipitation buffer: 1% CTAB, 50 mM Tris-HCl, pH 8.0, 10 mM Na_2EDTA .
10. TES buffer: 10 mM Tris-HCl, pH 8.0, 1 mM Na_2EDTA , 1 M NaCl.
11. 3 M Na_2 Acetate, pH 5.
12. TE buffer: 10 mM Na_2EDTA , 10 mM Tris-HCl, pH 8.0.
13. Proteinase K (United States Biochemical, Cleveland, OH).

2.3. UV-Endonuclease Alkaline-Sucrose-Sedimentation Analysis

1. Internal DNA size standards: Linear-dsDNA from bacteriophages T7 (40-kbp in virions), λ (50-kbp) and T4 (166-kbp) may be radiolabeled by growth in the presence of [^{14}C] thymidine in thymine-requiring bacteria, or in wild-type bacteria with deoxyadenosine added, and phage DNA isolated by standard techniques (*see*, for example, ref. [5]).
2. UV endonuclease: Both well-known CPD-glycosylase/AP-lyase enzymes, the so-called endonuclease V from *E. coli* bacteriophage T4 (TEV), and the enzyme from the bacterium *Micrococcus luteus*, have proven equally satisfactory for CPD assays. The *M. luteus* enzyme is available commercially from Applied Genetics, Inc., Freeport NY. Alternatively, it may easily be purified free from significant DNase activities (UV endonucleases are active in the presence of EDTA, unlike most DNases) from frozen cells (6). The phage T4 enzyme may be obtained commercially from Epicentre Technologies, Madison WI, or purified from *E. coli* overproducing it, as described by Manuel et al. (7).
3. UVE buffer: 10 mM Tris-HCl, pH 7.6, 20 mM Na_2EDTA , pH 7.6, 50 mM NaCl.
4. 1 M NaOH, freshly prepared.
5. 5–20% Alkaline (0.1 M NaOH) sucrose gradients, freshly prepared. Five milliliters are required if a Beckman SW 50.1 rotor is used. Other rotors (tubes) will require different volumes.

3. Methods

3.1. Radiolabeling In Vivo and Extraction of *Arabidopsis* DNA

The following procedure has been successful in our laboratory (3). The DNA extraction procedure is that of Rogers and Bendich, with minor modifications (8).

1. Grow *Arabidopsis* plants to the 8- to 10-leaf stage in MSS-agar containing 2 $\mu\text{Ci/mL}$ [^3H] thymidine. This typically yields plant DNA labeled at 3×10^4 cpm/ μg .
2. To extract DNA, grind 0.5 g of stem-leaf material under liquid N_2 .
3. Suspend the ground material in 0.5 mL of tissue resuspension buffer in a microcentrifuge tube and heat at 65°C for 1–3 min.
4. Add 50 ng of yeast tRNA.
5. Extract with an equal volume of chloroform:isoamyl alcohol (24:1). Sediment at 11,000g for 30 s in a microcentrifuge.
6. Re-extract the isolated upper layer with 0.1 vol of 65°C CTAB salt solution and 0.9 vol of chloroform:isoamyl alcohol. Sediment as above.
7. Mix the isolated top layer with 1 vol of CTAB precipitation buffer, and spin in a microcentrifuge for 10–60 s.
8. Resuspend the pellet in TES buffer (with heating for 5–10 min at 65°C if necessary), and precipitate the nucleic acids with 0.1 vol of 3 M sodium acetate plus 2 vol of ethanol.
9. To prevent accumulation of single-strand breaks during storage, treat the DNA preparation with 0.3 mg/mL proteinase K for 1 h.
10. Extract with 1 vol of phenol in the presence of 0.1% sodium dodecyl sulfate. Precipitate the DNA with ethanol-sodium acetate as in **step 8** and redissolve in 1/10 vol of TE.
11. DNA concentrations may be determined by the dye-fluorescence method of Labarca and Paigen (9). Typical yields from 0.5 g of material are 1.5–2.5 μg of DNA, average size 50 kb. (See **Notes 1** and **2**.)

3.2. Treatment of Plant DNA with UV Endonuclease

1. Prepare 100- μL samples containing at least 4000 cpm [^3H]DNA (ideally 10–20,000 cpm) from irradiated plants and unirradiated control plants in UVE buffer, and treat with excess UV endonuclease (determine this empirically, using the most heavily irradiated sample and two concentrations of enzyme). Omit endonuclease from parallel control samples.
2. Incubate all samples at 37°C for 90 min, and then add 10 μL of 1 M NaOH.

3.3. Sedimentation

1. Mix samples with about 3000 cpm of [^{14}C]DNA marker (e.g., phage λ DNA; see **item 1**, Subheading 2.3.) and layer onto a 5-mL 5–20% alkaline sucrose gradient.
2. Sediment as necessary to clear radioactivity from the top three fractions without pelleting significant amounts on the tube bottom, typically 3 h at 45,000 rpm in a

Beckman SW 50.1 rotor (243,000g) for irradiated, endonuclease-treated DNA. Control samples—unirradiated DNA, endonuclease-digested or mock-digested, irradiated but mock-digested—may require shorter sedimentation times. Collect about 30 fractions (*see* **Note 3**) and determine the radioactivity profile (*see* **Note 4**).

3.4. Analysis

The apparent number of ESS (therefore CPDs) per nucleotide of DNA equals $1/X_n - 1/X_{o,n}$, where X_n and $X_{o,n}$ are the number-average numbers of nucleotides (degrees of polymerization) in single-stranded DNA after and before UV endonuclease treatment, respectively. If it can be assumed that the DNA fragments analyzed correspond approximately to a random distribution about a mean, typically the case for small fragments derived from relatively large DNA, then the corresponding weight-average experimental determinations may be used, assuming $X_n = 0.5 X_w$ and $X_{o,n} = 0.5 X_{o,w}$. X_w averages, obtained by inserting the data from the n fractions into the formula $X_w = \Sigma X_{w,i} (\text{cpm})_i / \Sigma (\text{cpm})_i$, are less sensitive to very small DNA fragments, whose apparent velocities may be artifactual, than are X_n averages. $([\text{cpm}]_i)$ is the radioactivity in the i th fraction, and X_i values are determined as described below).

The numbers of the various fractions (1,2,3 . . . counting from the meniscus downward), relative to the number of the peak fraction(s) marking the positions of the [^{14}C]DNA size standard(s), correspond directly to the ratios of the average sedimentation coefficients S_i of the molecules in the i th fraction to the coefficient(s) $S_{o(j)}$ of the standard(s). These are related to the degrees of polymerization by an equation of the form $X_i = a S_i^b$, where a and b are empirical constants independent of X_i . Thus, $X_i/X_j = (S_i/S_j)^b$. If two or more DNA size standards are used, b may be calibrated from their positions (fraction numbers) in the gradient. If only a single size standard is available, b may be taken as 0.35, a value shown to fit alkaline-sucrose-gradient data for a very wide range of DNA sizes (**10**).

4. Notes

1. DNA molecular sizes may be estimated by sedimentation in neutral sucrose gradients in the presence of ^{14}C -labeled phage λ -DNA. If sizes are significantly smaller than the expected average distance between CPDs, precautions to prevent shearing of DNA during handling, such as use of constant-diameter pipets instead of conical disposable pipeter tips, should be employed.
2. Quaiter and coworkers (**11**) have described more elaborate techniques for gentle cell disruption (suitable for exposure of plant cell DNA to UV endonucleases), which can yield DNA well in excess of 100 kb.
3. Fraction sizes must be constant for the above analysis to hold, so a positive-displacement method of fraction collection is advisable.

4. Accurate determination of ^3H radioactivity in fractions containing ^{14}C requires correction for "spillover" of ^{14}C into the ^3H channel. The channel windows should be chosen so that spillover of ^3H into the ^{14}C channel is negligible, and a ^{14}C standard, in a mock sucrose-gradient fraction, should be used to determine the fraction counting in the ^3H channel.

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DNA Damage Quantitation by Alkaline Gel Electrophoresis

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1. Introduction

Physical and chemical agents in the environment, those used in clinical applications, or encountered during recreational exposures to sunlight, induce damages in DNA. Understanding the biological impact of these agents requires quantitation of the levels of such damages in laboratory test systems as well as in field or clinical samples. Alkaline gel electrophoresis provides a sensitive (down to ~ 2 lesions/5 Mb), rapid method of direct quantitation of a wide variety of DNA damages in nanogram quantities of nonradioactive DNAs from laboratory, field, or clinical specimens, including higher plants or animals. This method stems from studies of velocity sedimentation of DNA populations, and from the simple methods of agarose gel electrophoresis. Over the last ~ 15 years, our laboratories have developed quantitative agarose gel methods, analytical descriptions of DNA migration during electrophoresis on agarose gels (1,2), and electronic imaging for accurate determination of DNA mass. Although all these components improve sensitivity and throughput of large numbers of samples (3,4), a simple version using only standard molecular biology equipment allows routine analysis of DNA damages at moderate frequencies.

Damages can be measured in most linear DNAs, such as those from viruses (5), bacteria, simple eukaryotes, higher plants (6–11), and higher animals, including human tissues (12–17). For each species, the isolation procedure must be verified to yield DNA of suitable size and purity. In the gel method, sensitivity (lower limit of lesion frequency measurable) depends directly on the DNA size, and thus the larger the experimental DNA, the greater the sensitivity of lesion measurement. For lesions other than frank strand breaks, cleavage by a lesion-recognizing enzyme is required for lesion quantitation; sample DNAs must be

free from contaminants that interfere with enzyme cleavage at lesion sites or produce extraneous cleavages at nonlesion sites. The exact experiment to be carried out will depend on the question being asked: What is the level of damage induced by a certain concentration of chemical or dose of radiation? How efficiently does cell type A remove those damages relative to cell type B, and so on. It is beyond the scope of this chapter to discuss planning and execution of all such experiments; we will use as an example the quantitation of cyclobutane pyrimidine dimer (CPD) induction in cultured human cells at increasing ultraviolet (UV) light exposures. We present here a description of the methods, as well as a brief description of the underlying principles, required for a simplified approach to quantitation of DNA damages by alkaline-gel electrophoresis.

2. Materials

All solutions for DNA isolation, cleavage, and gel electrophoresis should be sterilized by appropriate means. Gels should be handled using powder-free gloved hands.

2.1. UV Irradiation and Sample Processing

1. Low-pressure Hg lamp: emits principally 254-nm (UV-C) wavelengths; for samples with little shielding (e.g., monolayer of cultured human cells).
2. Meter for 254-nm UV (*see Note 1*).
3. Red bulbs for room illumination (GE Party Bulb, 25 W red) (*18*).
4. Plumb line.
5. Human cells and materials for cell culture.
6. Phosphate-buffered saline: 0.17 M NaCl, 3.4 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄.
7. L buffer: 20 mM NaCl, 0.1 M EDTA, 10 mM Tris-HCl, pH 8.3.
8. L buffer containing 0.2% *n*-lauroyl sarcosine (Sigma, St. Louis, MO).
9. TE (Tris-EDTA) buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
10. Agarose for sample embedding (SeaPlaque or InCert agarose, FMC, Rockland, ME).
11. Proteinase K: 10 mg/mL stock in 10 mM Tris-HCl, pH 7.5. Prepare proteinase K solutions at 1 mg/mL in TE, and in 10 mM Tris-HCl, pH 7.5, 1 mM CaCl₂. Then predigest solutions for 1 h at 37°C. Check for endonuclease activity (integrity of supercoiled DNA); incubate supercoiled DNA with proteinase K solutions at 37°C for 1 h and overnight in both buffers. If satisfactory, purchase large quantities of that lot. Prepare 10 mg/mL stock in L buffer with 1% sarcosyl (for cells) or 2% sarcosyl for tissues.
12. Phenylmethylsulfonyl fluoride (PMSF): 40 mg/mL in isopropanol; store at -20°C.
13. *Micrococcus luteus* UV endonuclease or T4 endonuclease V (store in 40% glycerol at -20°C) (*see Note 2*).
14. Endonuclease buffer: 30 mM Tris-HCl, pH 7.6, 1 mM EDTA, 40 mM NaCl.

15. Endonuclease buffer containing 0.1 mM dithiothreitol (DTT), and 0.1 mg/mL bovine serum albumin (BSA) (New England Biolabs, Beverly, MA) made fresh.
16. Molecular-length standards (*see* **Notes 3** and **4**): uncut λ DNA; *Hind*III-digested λ DNA. (Aliquot into single-use portions, and store at -20°C .)

2.2. Alkaline-Gel Electrophoresis and Gel Processing

1. Bio-Rad (Hercules, CA) Mini-Sub Cell gel electrophoresis apparatus (*see* **Note 5**).
2. Tray for 6.5 cm $\times$ 10 cm gel. The Plexiglas gel tray should be cleaned thoroughly with hot water and detergent (and checked that it does not produce fluorescent residues) immediately after last use.
3. Comb for tray (15-well).
4. Power supply: Hoefer (Pharmacia, Piscataway, NJ) PS250/2.5 A or equivalent.
5. Pump for buffer recirculation.
6. Cooling bath for immersion of electrophoresis apparatus.
7. LE agarose (FMC) (*see* **Note 6**).
8. Deionized, double-distilled water.
9. 5 M NaCl.
10. 0.1 M EDTA, pH 8.0.
11. Time Tape (TimeMed Labeling Systems, Inc., Burr Ridge, IL).
12. Plastic ruler or spacer (0.02 inch thick).
13. Alkaline electrophoresis solution: 30 mM NaOH, 2 mM EDTA (**19**).
14. Leveling plate and small spirit level.
15. Dust cover (plastic shoebox).
16. 70% ethanol.
17. Lint-free tissue.
18. Microwave oven.
19. Alkaline stop mix: one part alkaline dye mix (0.25% bromocresol green in 0.25 N NaOH, 50% glycerol): one part 6 N NaOH.
20. Disposable bacteriological loops (1 μL , USA Scientific, Ocala, FL).
21. Ethidium bromide: Prepare a 10 mg/mL ethidium bromide solution using double-distilled water. Stir the solution using an electric stirring motor and stir bar until the ethidium is well dissolved. Filter through a 0.2- μm filter, and subdivide into portions appropriate to ~ 1 wk of use. Keep one tube (capped and wrapped with foil) at room temperature; store stock at -20°C .

Ethidium bromide is a mutagen. Investigators should wear gloves, and handle the solution as a potential hazard. Ethidium is also light-sensitive; keep the stock solution in subdued light.

22. Stainless-steel or glass pan.
23. Vinyl, powder-free gloves.
24. Suction apparatus with water trap.
25. Gel platform rocker, variable speed (Bellco, Vineland, NJ).
26. 1 M Tris-HCl, pH 8.0.

2.3. DNA Visualization and Quantitation

1. UV transilluminator.
2. Polaroid camera system and Polaroid type 55 P/N film.
3. Densitometer.
4. Step density wedge.

3. Methods

3.1. Preparation of Alkaline Agarose Gel

1. Rinse the leveling plate with distilled water, then 70% ethanol, and dry with lint-free tissue. Wipe the gel tray and comb with ethanol using a lint-free tissue.
2. Neatly tape the open ends of the gel tray with Time Tape press firmly to seal. The tape under the tray must be flat and even. Adjust the comb to the proper height for the gel tray. Store the tray and comb under the clean dust cover.
3. Place 50 mL of H₂O in a 250-mL bottle; place ~100 mL of H₂O into a second 250-mL bottle.
4. Add 0.4 g of LE agarose to the first bottle. **Do not cap the bottles (Hazard).**
5. Microwave both bottles on high (650 W) for 8 min; watch to prevent liquid overflow or excess evaporation. Add additional warm water to the agarose solution if necessary.
6. Pour the warmed water into a clean, dust-free, sterile graduated cylinder.
7. Add ~20 mL of warm water to the agarose solution and swirl; add 1 mL of 5 M NaCl, 0.1 mL of 0.1 M EDTA (per 100 mL final volume), and swirl to mix.
8. Discard the water from the warmed cylinder.
9. Pour the agarose solution into the warmed cylinder; bring to 100 mL with heated water. Pour the agarose back into the (empty) warm bottle, and swirl to mix. Inspect the agarose solution for incomplete dissolution of agarose, or dust, fibers, or other particles.
10. The agarose solution may be capped and placed in a 55°C bath for no more than 2 h; discard if the solution becomes inhomogeneous.
11. Using the warmed (or rewarmed, if necessary) cylinder, measure the required volume of agarose (35 mL/6.5 cm × 10 cm gel). With the gel tray on the leveling plate, remove the comb from the tray. Pour the agarose slowly into the gel tray. Reset the comb exactly perpendicular to the long axis of the gel tray.
12. Replace the dust cover over the gel, and allow to set ~1 h (0.4% gel, room temperature).
13. Pour cold electrophoresis solution over the gel; pick up the comb at one edge, and then remove the rest of the comb.
14. Cover the gel with electrophoresis solution (prevents well collapse, equilibrates gel).
15. Transfer the gel to the apparatus (preleveled and checked for solution recirculation) containing chilled electrophoresis solution; equilibrate ~1 h by recirculating the electrophoresis solution. Set the apparatus on black paper to aid visualizing the wells.

3.2. UV Irradiating and Processing the Sample

1. Melt Sea Plaque or InCert agarose (2% in TE), and place at 45°C.
2. Turn the UV lamp on ~15 min before use; after warmup, wrap the end ~3 in of the bulb with foil. **UV-C is an eye hazard; wear UV-opaque glasses with side shields.**
3. Use a plumb line to locate the position for cell irradiation exactly under the bulb. Take care that cells at the periphery are not shaded by the sides of the dish.

The following steps should be carried out under red light illumination to prevent photorepair.

4. Remove the medium from the cells, and rinse two to three times with ice-cold PBS. Keep the cells cold to minimize repair. Irradiate suspension cells in PBS at low optical density. (Do not irradiate cells in a narrow tube from above—inaccurate dosimetry.)
5. Immediately after UV treatment, suspend the cells in PBS at a concentration of 10^6 human cells/mL.
6. Mix 1 mL of cells at 2×10^6 cells/mL with 1 mL of agarose.
7. Pipet 10- μ L aliquots of suspension into “buttons” onto a Petri dish on ice. Let solidify.
8. Immerse the buttons immediately in proteinase K solution, transfer to a multiwell dish or 35-mm suspension culture dish, seal with parafilm, and incubate at 37°C.
9. Replace the proteinase K solution daily for 4 d.
10. Check for complete removal of proteins by electrophoresing DNA on 0.4% alkaline-agarose gels (rinse the buttons with TE, and denature; *see steps 19–21*). If DNA remains at the well-gel interface, digestion is incomplete; after adequate removal of cellular proteins, DNA samples will electrophorese readily into an alkaline gel.
11. Treat samples showing incomplete digestion with proteinase K as above.
12. Rinse the buttons twice with ice-cold TE, twice with 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 40 μ g/mL PMSF at 45°C for 1 h, and then rinse with TE.
13. Store the buttons at 4°C in L buffer containing 2% sarcosyl.
14. Wash the buttons in 5 vol of ice-cold TE, and soak twice in 5 vol of TE for 20 min each.
15. Transfer to lesion-specific endonuclease buffer, and incubate in two changes for 1 h.
16. Transfer to endonuclease buffer containing 0.1 mM DTT and 0.1 mg/mL BSA. Use companion buttons (replicate buttons from each experimental sample) for each dimer determination. Incubate the samples on ice for at least 60 min.
17. Calculate the quantity of UV endonuclease for the “+ endonuclease” sample from the endonuclease activity (*see Note 2*), the quantity of DNA per button, and the maximum expected CPD level. **The validity of the assays depends on cleavage at all lesion sites; sufficient endonuclease must be used to give complete cleavage. (See Note 7.)**
18. In the “+ endonuclease” sample, replace the buffer with buffer plus endonuclease. Add buffer without endonuclease to the “– endonuclease” sample. Incubate the

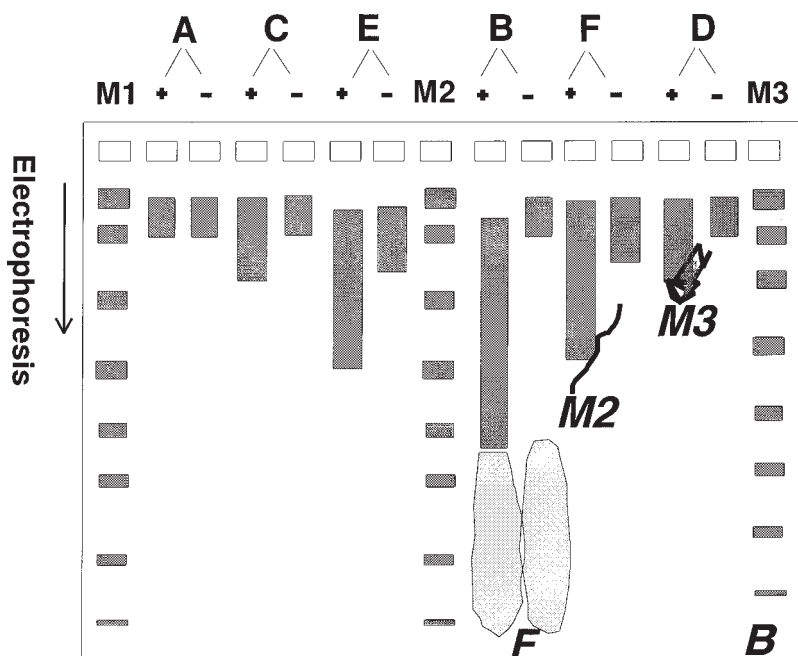


Fig. 1. Schematic diagram of an alkaline electrophoretic gel for DNA damage quantitation. Molecular-length standard DNAs (M1, M2, and M3) are shown in lanes 1, 8, and 15. In the experiment shown, 6 experimental sample pairs (A, B, C, D, E, and F) are included on the gel. The “+ endonuclease” and “– endonuclease” members of each sample pair are placed in adjacent lanes, but (to avoid bias in analysis), the pairs are not necessarily arranged in experimental order. The italic labels refer to specific experimental problems frequently encountered. (See **Table 1**.)

samples on ice for 30 min, then transfer to 37°C and incubate for 60 min. At this time, prepare the molecular-length standards (*see steps 22 and 23*).

19. Rinse the buttons with TE.
20. Add 10 μ L of alkaline stop solution; incubate at room temperature for 30 min.
21. Rinse the buttons with alkaline electrophoresis solution just prior to loading onto the gel.
22. Dilute molecular-length standard DNAs into TE at ≤ 80 ng/ μ L (20).
23. Add alkaline stop solution (2 μ L/10 μ L DNA solution or button), and incubate the length standards under the same conditions as the experimental DNAs.

3.3. Sample Loading and Gel Electrophoresis

1. Buttons are loaded into wells with the gel on a bench top rather than in the apparatus. Remove the gel and tray from the apparatus; place on a clean, lint-free tissue. Protect the gel surface by covering with plastic wrap or film.
2. For a 15-well gel, use lanes 1, 8, and 15 for molecular-length standards (*see Fig. 1*), leaving 12 lanes for 6 sample pairs. The “+” and “–” endonuclease samples of each

pair are placed in adjacent lanes; to avoid bias in analysis, code experimental sample pairs; place members of different pairs at coded locations on the gel.

3. Place the tubes containing the samples close to the gel.
4. Pick up individual buttons from the solution using a plastic disposable loop, and deposit each button in a well (containing alkaline electrophoresis solution); it should slip readily into the well. Generally buttons are not sealed into the wells; however, ~5–15 μL of 0.4% agarose may be micropipetted into each well so that buttons do not become displaced.
5. If the molecular-length standard DNAs are formed into buttons, load them in the gel along with the experimental buttons. If the standards are in solution, they should be loaded after the gel is replaced in the electrophoresis apparatus. Replicate length standards are in lanes 1, 8, and 15.
6. Set up the Mini-Sub cell apparatus for buffer recirculation. Fill the apparatus with 250 mL of prechilled alkaline electrophoresis solution (*see Note 8*). Before inserting the gel, check that the solution circulates and the tubing does not leak.
7. After the gel tray and all samples are inserted, check the apparatus with a spirit level, and level if necessary before electrophoresis is begun.
8. Begin electrophoresis (~1.5 V/cm; the value depends on DNA size) for 30 min without recirculation of electrophoresis solution.
9. Start recirculation of the electrophoresis solution, and continue throughout the electrophoresis. Use a timed, voltage-controlled power supply to electrophorese for the correct period.
10. After electrophoresis, remove the gel and tray from the apparatus, and process the gel as described in **Subheading 3.4.**, below.
11. Immediately after electrophoresis, remove the electrophoresis solution from the apparatus (alkaline solution is corrosive to electrodes) and discard it. Rinse the apparatus and tubing thoroughly, and invert on lint-free tissue in a dust-free location to dry.

3.4. Gel Neutralization and DNA Staining

1. After electrophoresis, remove the gel and tray from the apparatus (the alkaline solution makes the gel slick, so take care the gel does not slide out of apparatus onto the floor). Wear powder-free vinyl gloves to protect hands, and to protect the gel from fingers. 0.4% gels are fragile; handle carefully.
2. Rinse the gel surface (while the gel is in the gel tray) in a gentle stream of distilled water.
3. Gently transfer the gel to a pan.
4. Carefully add water to the pan, at a position away from the gel.
5. Rock the pan gently, and then remove the water using a suction device (holding the suction device away from the gel).
6. From stock 1 M Tris-HCl, pH 8.0, make 500 mL of 0.1 M Tris-HCl neutralizing solution.
7. Pour 250 mL into the pan, away from the gel; place the pan on gel rocker for ~20 min.
8. Carefully remove the neutralizing solution.

Table 1
Troubleshooting Quantitative Agarose Gels

Problem	Possible Cause(s)	Solution(s)
A. No DNA visible	Sample not loaded	Load sample
	Insufficient DNA loaded	Load more DNA
	Nuclease degradation of DNA	Discard degraded DNA
	Ethidium bromide photobleached	Use fresh ethidium
	Electrophoresis polarity reversed	Reverse polarity
B. Gel lanes crooked	Gel not level during pouring	Use leveling plate
	Gel rig not leveled	Use spirit level
	Thermal currents over rig	Place box over rig
C. DNA “smiles”	Wells collapsed	Remove comb, add
	Wells dried out	buffer to wells and over gel
D. DNA migration depends on amount of DNA	DNA too concentrated	Dilute DNA samples
E. DNA lanes slant in photograph	Comb crooked when gel poured	Align comb precisely
	Gel photographed at slant	Check that marker lanes are exactly parallel and straight
F. “Fuzzy” cloud of ethidium-stained material near lane bottom	RNA from sample	RNase sample
	RNA from endonuclease	RNase endonuclease
G. “Unirradiated” sample cleaved by endonuclease	Sample actually was irradiated	Check sample history
	Endonuclease contains non-specific cleaving activity	Use better endonuclease
	Non-sterile buffer, tube, tip	Use freshly sterilized buffer, and so forth
H. “Minus endo” sample degraded	Poor extraction method or technique	Evaluate method
	Non-sterile buffer, tube, tip	Use freshly sterilized buffer, and so forth
I. DNA length standards contain extra bands	Incomplete restriction digest	Carry out new digestion; check completeness of digestion
J. DNA length standards missing bands	Wrong DNA or restriction enzyme	Check DNA and enzyme
	Smaller bands electrophoresed off end of gel	Use shorter electrophoresis time or lower voltage

Table 1 (continued)

Problem	Possible Cause(s)	Solution(s)
K. High background fluorescence on gel	Too much ethidium in staining solution	Check ethidium stain
	Bacterial contamination in agarose solution	Make fresh agarose
	Agarose contains DNA contaminant	Use high quality agarose
	Agarose prepared from solution with bacterial/fungal/viral contaminant	Discard solutions, use freshly prepared
L. Gel will not set	Wrong agarose used	Use agarose intended for gel electrophoresis
	Dry agarose stored in moist conditions; has adsorbed water from atmosphere	Store agarose powder in presence of dessicant
	Agarose incompletely melted	Melt agarose thoroughly
M. Fluorescent particles on gel:		
1. Specks	Dust in agarose solution or in gel	Use filtered solution
	Dust on gel	Cover gel
2. Strands	Lint in agarose solution	Dry glassware on lint-free wipe
	Wipe gel apparatus, trays with lint-free wipe	
3. Globs	Ethidium aggregates on gel	Filter ethidium stock
	Discard working ethidium solution, use fresh	

9. Add 250 mL of fresh 0.1 M Tris-HCl, and neutralize the gel for at least 40 min. For high-molecular-length DNAs that diffuse slowly, the gel can be neutralized overnight. (*See Note 9.*)
10. Prepare the stain (250 mL of 1 µg/mL ethidium bromide in ddH₂O) in a clean, dust-free cylinder.
11. Remove the final neutralizing solution, and pour the ethidium solution into the pan, well away from the gel. Do not pipet stock ethidium solution just above the gel surface, since this produces uneven gel staining. Stain the gel for 15 min.
12. Remove the ethidium solution, and rinse the gel gently with double-distilled water.
13. Fill the gel pan (~2/3 full) with water, and destain the gel for at least two changes, for 15 min each (*see Note 10*).

3.5. DNA Visualization and Quantitation

For number average length calculations, we need to know the position of DNA molecules on the gel and the quantity of DNA at each position. We need know only relative—not absolute—masses of DNA molecules of different sizes in different lanes. Thus, with uniform gel, ethidium background and transilluminator, and DNA staining uniform across the gel (dependent only on DNA mass), we need a recording system giving a signal proportional to DNA mass. Photographic film is widely used for recording fluorescence from DNA, but its response to fluorescence is linear over a very limited range, determined by DNA concentrations, gel conditions and photographic conditions (film type, temperature of storage and use, exposure, processing). *See (21)* for a discussion.

To determine the linear range for specific experimental conditions, prepare a standard alkaline agarose gel, and electrophorese increasing DNA masses (a few to several hundred nanograms per lane) in different gel lanes. Electrophorese and process the gel as usual; photograph the gel, scan the DNA lanes recorded on the film with a densitometer, and determine the relation of quantity of DNA to densitometric response (“area” of each band). Plot DNA quantity vs “area” of that band, noting the threshold, linear response range, and saturation. In all damage determinations, use DNA concentrations within the linear range.

3.5.1. Photography of Ethidium Fluorescence on Electrophoretic Gels

1. Place the neutralized, stained, and destained gel on the transilluminator. If the transilluminator is uneven (i.e., shows “stripes” corresponding to the lamps), orient the gel so that illumination down a lane is constant.
2. Photograph the gel with film generating a negative. Do not attempt to obtain quantitative data from a positive print, since its darkening (measured in reflectance) does not represent reliably the fluorescence to which it was exposed.
3. Process the film according to standardized conditions (*see Subheading 3.5., above*).
4. Dry in a dust-free environment. Streaking or fingerprints on the negative interfere with accurate DNA mass quantitation.

3.5.2. Densitometry

Test the densitometer’s linearity of response to film darkening:

1. Align the gel precisely on the transilluminator. Since DNA migration is a function of its molecular length, the film must be aligned precisely so that an x position on the densitometer trace uniformly represents DNA migration in all lanes. Align same-sized molecular-length standards in different gel lanes at the same x migration position on the densitometer trace.
2. Obtain traces (intensity of fluorescence as a function of migration position on the gel) for each molecular-length and experimental sample lane. For densitometers with computer output, data may be stored and the quantitative values used

for further manipulations. However, analog outputs (traces of DNA mass as a function of lane migration) can also be used.

3.6. Theory of Analysis

Suppose that an initial DNA population contains N_0 molecules, and k strand breaks are introduced directly (e.g., by X-rays) or by lesion-specific endonucleases. Each strand break increases the total number of DNA molecules by one, resulting in a final population of $N_+ = N_0 + k$ DNA molecules. To determine the number of strand breaks, we count the number of DNA molecules before and after introduction of the breaks, i.e., $k = N_+ - N_0$. Although this theory is simple, there are problems with implementation. First, we must count DNA molecules; accuracy in this simple counting approach would require samples of exactly the same size, which is never easy. Normalizing by the total mass of DNA avoids both problems.

3.6.1. "Normalizing" Removes the Need for Samples of Equal DNA Mass

Rather than determining the number of molecules, we determine the number of molecules per unit mass of DNA. This ratio is not changed by variations in the sample size if the sampled material is homogeneous. We could express the DNA mass in a variety of units. The most useful is the total number of individual bases or basepairs. (We use bases if we are measuring single-strand breaks and basepairs for double-strand breaks. In all that follows, "or basepairs" is implied whenever we give DNA masses in "bases".) We can imagine assigning an index number, i , to each DNA molecule, and determining its length in bases. If L_i represents the length (mass) of that molecule and if there are N DNA molecules in the sample, then the total mass of DNA is $\sum_i L_i$ where i goes from 1 to N . Our measure of strand breaks is $N / \sum_i L_i$. The units are "molecules per base," but we usually express DNA mass in some multiple of bases, therefore giving normalized values of, e.g., molecules per megabases. The reciprocal of the molecules per base is the average number of bases per molecule. Formally, this is called the number average length of the population, $\langle L \rangle_n$. From the definitions given above, $\langle L \rangle_n = (\sum_i L_i) / N$. Inducing breaks increases the number of molecules and decreases their average length. Our measure of the breaks produced by a given treatment is the number of breaks per unit length of DNA, i.e., the frequency of strand breaks, which is expressed in terms of number average lengths by the equation:

$$\phi = \langle L \rangle_{n,+}^{-1} - \langle L \rangle_{n,0}^{-1}, \quad (1)$$

where the subscripts 0 and + indicate initial and final (untreated and treated) populations, respectively.

3.6.2. Determining the Number of DNA Molecules Per Unit DNA Length by Gel Electrophoresis

Fluorescence from ethidium bromide is directly proportional to the mass of each molecule. That is, $f_i = k L_i$, where f_i is the fluorescence from molecule i , and k is a constant of proportionality that depends on many experimental factors. Mass normalization eliminates the need to determine the value of k as long as it is the same for all DNA molecules in a sample (i.e., lane of a gel). Instead of determining $\langle L \rangle_n$ by summing over i , suppose we separate the DNA molecules as a function of length, e.g., by gel electrophoresis. If n_L is the number of molecules in a sample of length L , then the total fluorescence owing to all the molecules of this length, f_L , is given by $f_L = k n_L L$. The total number of DNA molecules in the sample is $\sum_L n_L$ and the total number of bases in the same sample is $\sum_L n_L L$, where the sums extend from 1 to the number of bases in the largest DNA molecule. In terms of these sums, $\langle L \rangle_n = [\sum_L n_L L] / [\sum_L n_L]$. We can replace n_L by $f_L / (k L)$ and the product $n_L L$ by f_L / k . Thus, if k is the same for all molecules in the sample:

$$\langle L \rangle_n = \frac{\sum_L f_L}{\sum_L \frac{f_L}{L}}.$$

This equation for $\langle L \rangle_n$ indicates a sum over all values of L , the length of the DNA molecules, but we obtain the distribution of the DNA as a function of e.g., the distance of travel during electrophoresis. Although DNA lengths can only be discrete (integer) values, the distances moved by molecules of different lengths are continuous values (real numbers). Thus, the sums in the expression for $\langle L \rangle_n$ are replaced by integrals:

$$\langle L \rangle_n = \frac{\int f(x) dx}{\int \frac{f(x)}{L(x)} dx}, \quad (2)$$

where $f(x) dx$ is the intensity of fluorescence from a region of width dx at location x , while $L(x)$ is the length of the DNA molecules at this position, and x can be thought of either as the migration distance or more generally a "separation coordinate." The limits of integration must span the values of x for which there is measurable DNA. $L(x)$ is called the dispersion function of the separation system and is treated here as a continuous function of x . The actual values of x never appear explicitly in the equation for $\langle L \rangle_n$, only the values of f and L associated with given values of x . Thus, we can express x in any convenient

units. For digital data, “pixels” are a good choice. Because pixels divide the data into discrete intervals, the integrals in the equation for $\langle L \rangle_n$ revert (approximately) to sums. Although it is convenient to think of x as the migration distance, it is actually just a particular position on the gel along the direction of electrophoresis. Therefore, we can choose any origin for the x -axis, not just the lower edge of the loading wells.

We can either determine the dispersion function empirically, or obtain analytical functions that describe it. The method described in **Subheading 3.7.1.**, uses an empirical dispersion function, but analytical dispersion functions facilitate calculation of $\langle L \rangle_n$ directly from **Eq. 2**. For both static field and unidirectional pulsed-field (22) gel electrophoresis, the dispersion function is reasonably approximated by a hyperbolic function (23), which is specified by three constants that must be determined for each gel from observed distances of migration of DNA molecules of known length. As originally presented (23), these parameters were arbitrary fitting constants. By rearranging the hyperbolic equation, we obtained equivalent, physically meaningful constants (1,2). The hyperbolic dispersion function can be given by:

$$\frac{L(x)}{L_m} = \frac{x_0 - x}{x - x_\infty}$$

where x_0 and x_∞ are the locations on the gel of (hypothetical) molecules of “zero” and “infinite” length, respectively, and L_m is the length of the molecules that migrated to a position exactly halfway between x_0 and x_∞ . (That is, $L_m = L[x_m]$, where $x_m = [x_0 + x_\infty] / 2$). Once the values of x_0 , x_∞ and L_m are known for a particular gel, we can compute $L(x)$ for every value of x between x_0 and x_∞ . Although the physically meaningful parameters shown above are conceptually appealing, the sets of constants given by Southern (23) or in our previous work (1) are equivalent for computing dispersion functions. The three parameters can be determined either by nonlinear fitting or a linear least-squares method (24). All such methods require a data set containing the distances of migration and length of at least three DNA length standards, although many more are desirable.

3.6.3. Alternate Determination of Number Average Length: Median Length

The presence of $L(x)$ in the denominator of **Eq. 2** for $\langle L \rangle_n$ can produce experimental difficulty when the length approaches very small values. As long as most of the DNA is large, the fluorescence (or other label) will not give a significant signal for positions on the gel corresponding to small DNA molecules, and the integration can be truncated before x gets too close to x_0

(which causes $L(x) \rightarrow 0$). For DNA with many strand breaks, there may be significant signals, for values of x near x_0 . For such cases, we can obtain approximate values of the number average length of the population from either the length average length or the median length (**I**). Median length, L_{med} , is the length of the DNA molecules that migrate to the position x_{med} , the value of x that divides the mass of DNA exactly in half. Formally, we can define the median distance of migration of a DNA sample from the equations:

$$\int_{x_{\infty}}^{x_{med}} f(x) dx = \frac{1}{2} \int_{x_{\infty}}^{x_0} f(x) dx,$$

and

$$L_{med} = L(x_{med}).$$

3.6.4. Relation of Median Lengths to Number Average Length

There are two special cases where there are known relationships between $\langle L \rangle_n$ and L_{med} . For a population of molecules all of which are exactly the same size, $\langle L \rangle_n = L_{med}$. If the population contains molecules of more than one length, L_{med} will be greater than $\langle L \rangle_n$, because larger molecules are weighted more heavily. The second special case is where each molecule in an initial homogeneous population has been broken randomly several times, as, for example, during extraction. A population of DNA molecules from higher organisms (where the initial length is the length of the chromosomes) that has been reduced in length sufficiently that the resulting distribution can be separated in a static-field gel should fit this requirement quite well. Under these conditions, the number average length of the population is given by the equation (25):

$$\langle L \rangle_n \approx 0.6 L_{med}. \quad (3)$$

Thus, the error associated with estimating the number average length of a population using the median length is never worse than a factor of 5/3, and in the common situation of DNA broken extensively during extraction, should be much better.

3.7. Obtaining Median Lengths and Calculating Lesion Frequencies

This discussion presumes access to molecular biology equipment, but not specialized equipment for high-sensitivity, high-throughput DNA lesion quantitation (alkaline pulsed-field gels, quantitative electronic imaging,

computerized analysis). In this simple approach, DNA median molecular lengths are calculated (5) and, from them, number average molecular lengths (25).

3.7.1. Determination of DNA Dispersion Function

1. Compare lane traces of molecular length standards. The peak positions of the DNAs of the same molecular length should exactly coincide. If so, one lane of standards establishes a DNA dispersion function for the entire gel. If the traces do not coincide, standard lanes near individual experimental samples should be used to calculate separate DNA dispersion in different gel areas.
2. Determine X, Y coordinates of each DNA length standard. (X corresponds to the migration position of the peak of a DNA band; Y is the molecular length of that DNA in basepairs.)
3. Plot these points on linear-linear scales.
4. Fit a curve through the data points. This DNA dispersion function relates size of DNA molecules to migration position on this gel. Since migration position is affected by exact electrophoresis conditions, DNA dispersion curves must be determined for each gel (or gel region; *see step 1 of Subheading 3.7.1.*).

3.7.2. Determination of Median and Number Average Molecular Lengths

The median molecular length is the molecular length in the middle of DNA mass, i.e., the molecular size of which one-half the DNA molecular mass is larger and one-half is smaller. The manual method described below indicates the calculation; it could also be done by a computer “area” computation.

1. Photocopy the DNA lane (photocopier paper is quite uniform).
2. Handle photocopies with powder-free gloves to ensure that neither oils, moisture from hands, nor powder from the gloves interferes with measurement.
3. Cut out the trace of an experimental DNA lane carefully with scissors.
4. Determine the weight (W) of the trace using an analytical balance; calculate $W/2$.
5. Estimate the x position (x_I) corresponding to the middle of the DNA mass; cut the trace vertically at x_I .
6. Weigh one of the resulting half-traces, yielding w_I .
7. If $w_I = W/2$, refer x_I to the dispersion plot, and determine the corresponding molecular length L_{med} , the median molecular length of that DNA population.
8. If $w_I \neq W/2$, gradually slice the larger half vertically until its weight equals $W/2$.
9. Locate the x position of this slice giving one-half the weight in that portion of the lane trace on the dispersion curve; the corresponding length value is the median molecular length, L_{med} .
10. Calculate the number average molecular length, $\langle L \rangle_n$, from **Eq. 3**.

3.7.3. Computation of DNA Lesion Frequency

Calculate the frequency of DNA lesions according to **Eq. 1**: where $\langle L \rangle_{n,+}$ is the number average length of the treated sample, and $\langle L \rangle_{n,0}$ is the number

average length of the untreated sample. For samples in which DNA lesions were revealed by lesion-specific agent cleavage, “treated” refers to samples treated with that agent, whereas “untreated” refers to the companion part of the sample not treated with the agent. This approach provides high sensitivity, since the experimental DNA is extracted, then split into samples for agent-specific cleavage. It also allows determination of levels of background lesions. For strand breaks induced directly by radiation, chemicals, and so forth, the “treated” sample is the one exposed to the radiation or chemical, and the “untreated” sample is the unexposed one. This determination is more difficult, since DNAs in samples to be compared are extracted independently. Reproducible isolation procedures are essential for accurate calculation of directly induced strand breaks.

3.8. High-Sensitivity Measurements

The methods described above (static-field electrophoresis, photographic recording of DNA mass, computation of median molecular length) will give quite adequate measurement of DNA damages down to $\sim 2/\text{Mb}$. We can compare that value to a relevant biological dose: the D_{37} for 254 nm exposure of mammalian cells is 7 J/m^2 , and 1 J/m^2 of 254 nm radiation induces about 6.5 CPD per million bases. Thus, the D_{37} induces 45 CPD/Mb, indicating that the gel method can readily measure responses within the range of high cell survival.

For higher-sensitivity measurement, three major changes are required: first, higher-molecular-length DNAs are needed; for methods of obtaining high-molecular-length DNA from various higher organisms, the reader is referred to references (8,26,27). Second, these large DNAs must be separated, readily carried out by pulsed-field electrophoresis (22,28–31). Third, a method of quantitating DNA with a linear response and large dynamic range (3,32) allows more accurate measurement of DNA mass, especially at the leading edge of the DNA peak, corresponding to the smaller molecules in the population. Fourth, computerized calculation of the number average molecular length, rather than through its estimation through calculation of the median molecular length, allows much higher sensitivity of lesion measurement.

4. Notes

1. Commercial UV meters have filters transmitting limited wavebands, with the meter output weighted to specific spectral distributions. Other spectral distributions will not be measured accurately, and radiation of wavelengths not transmitted by the filter will not be recorded. Thus, the output of a “UV-A” lamp reported by a UV-A meter may give an accurate measure of UV-A radiation, but this measurement will not reflect any UV-B also emitted from the lamp. UV-B radiation can be orders of magnitude more effective in inducing biological damage than UV-A (6,14).

2. Pyrimidine dimer-specific endonucleases include the *Micrococcus luteus* UV endonuclease and bacteriophage T4 endonuclease V (commercially available from Epicentre, Madison WI). Preparations must be checked for nonspecific nucleases (cleavage of supercoiled DNA without CPDs), as well as activity (CPD sites incised/volume/time in standard conditions, e.g., 4×10^{15} CPDs incised/ $\mu\text{L}/\text{h}$), or specific activity (CPDs incised/protein/time). Activities reported as “ μg irradiated DNA cleaved/unit protein/unit time” are not useful, since the level of dimers in “irradiated,” DNA depends on the UV wavelength, exposure, and DNA base composition.
3. DNA length standards should span the lengths of experimental DNAs. Static-field gel electrophoresis resolves only molecules <50 kb (**19**); thus standards should include DNAs of length at least 50 kb. Commercially available standards include λ DNA (48.5 kb), and *Hind*III-digested λ DNA (23.1, 9.4, 6.6, 4.4, 2.3, 2, 0.56 kb). Since DNA conformation affects mobility on electrophoretic gels, neither circular nor supercoiled DNAs should be used as molecular length standards for linear DNAs.
4. All DNA length standards should be checked for integrity on alkaline agarose gels. Commercial DNAs are usually evaluated on neutral gels; such gels do not reveal single-strand breaks that will interfere with the use of the DNA as a length standard on alkaline gels. (Detailed procedures for alkaline agarose electrophoresis are given in **Subheadings 3.1., 3.3., and 3.4.**) Electrophorese higher-molecular-length standard DNAs on a static field, alkaline, 0.4% agarose gel (along with other DNAs of previously verified size); neutralize the gel, stain with ethidium, destain, and photograph. The DNA should appear as a single band, with little evidence of heterodispersity from single-strand breaks. Evaluate restriction digests for integrity (as above) and for complete digestion on a static-field neutral gel: the number and sizes of bands should correspond to those expected. Incomplete digests contain partial digestion products, which may be confusing if their lengths are assigned incorrectly. Where photographic conditions provide a linear response to DNA mass, the mass of DNA in each band should be directly proportional to its length.
5. Measurement of voltage across a gel:
 - a. Drill two small holes (each large enough for insertion of a volt meter probe) a known distance (e.g., 10 cm) apart in the top cover of the gel apparatus above the two ends of the gel.
 - b. Place a gel of standard size and composition, and electrophoresis solution of standard composition and volume in the apparatus as usual.
 - c. Insert the probes into the gel and begin electrophoresis.
 - d. Read the voltage on the voltmeter; knowing the distance between the two probes, calculate the voltage/centimeter of gel. (Voltages measured between electrodes of the gel apparatus vary with the individual apparatus, and thus cannot be applied to a different apparatus, whereas voltages per distance of gel can be.)
6. Gels for quantitation must provide both a resolving medium to separate DNAs according to size, and an optical medium for accurate measurement of DNA mass

- (low background fluorescence, no extraneous particles, especially those fluorescing at the wavelengths emitted by the DNA binding fluorophore).
7. Check by incubating replicate buttons containing DNA with the highest damage levels—as well as undamaged DNA to check nonspecific cleavage with increasing quantities of endonuclease. Determine the quantity of endonuclease required for complete cleavage, and then add excess enzyme to each “+ endonuclease” sample.
 8. Temperature control: DNA migration, and thus resolution of DNA molecules, on electrophoretic gels can be affected by temperature. To achieve temperature uniformity, immerse the gel apparatus in a cooling, recirculating bath filled so that the cooling solution reaches the level of the gel within the apparatus, or set the apparatus in a pan of crushed ice water, taking care that ice does not fall into the electrophoresis solution; replenish the ice periodically. Level the gel in the cooling apparatus. This is a potential safety hazard! Take care that leads from the power supply do not become submerged in the cooling water!
 9. For complex DNAs, complete renaturation (i.e., restoration of the original double-stranded conformation) is not usual; the formation of hairpins, which still retain partially single-stranded character, is more likely.
 10. Additional destaining time (overnight for high-molecular-length DNAs) and fresh water reduce nonspecific ethidium background. Gels may be destained at 4°C; however, bubbles appearing in the gel during warming will interfere with DNA quantitation.

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The Comet Assay (Single-Cell Gel Test)

A Sensitive Genotoxicity Test for the Detection of DNA Damage and Repair

Günter Speit and Andreas Hartmann

1. Introduction

The comet assay or single-cell gel (SCG) test is becoming established as a useful technique for studying DNA damage and repair. In this microgel electrophoresis technique, a small number of cells suspended in a thin agarose gel on a microscope slide is lysed, electrophoresed, and stained with a fluorescent DNA binding dye. Cells with increased DNA damage display increased migration of chromosomal DNA from the nucleus toward the anode, which resembles the shape of a comet (**Fig. 1**). In its alkaline version, which is mainly used, DNA strand breaks and alkali-labile sites become apparent, and the amount of DNA migration indicates the amount of DNA damage in the cell. The comet assay combines the simplicity of biochemical techniques for detecting DNA single-strand breaks and/or alkali-labile sites with the single-cell approach typical of cytogenetic assays. The advantages of the SCG test include its simple and rapid performance, its sensitivity for detecting DNA damage, the analysis of data at the level of the individual cell, the use of extremely small samples, and its applicability to virtually any eukaryote cell population. Apart from image analysis, which greatly facilitates and enhances the possibilities of comet measurements, the cost of performing the assay is extremely low. The comet assay has already been used in many studies to assess DNA damage and repair induced by various agents in a variety of cells in vitro and in vivo (for a review, see **1,2**). The test has widespread applications in genotoxicity testing, DNA damage and repair studies, environmental biomonitoring, and human population monitoring.

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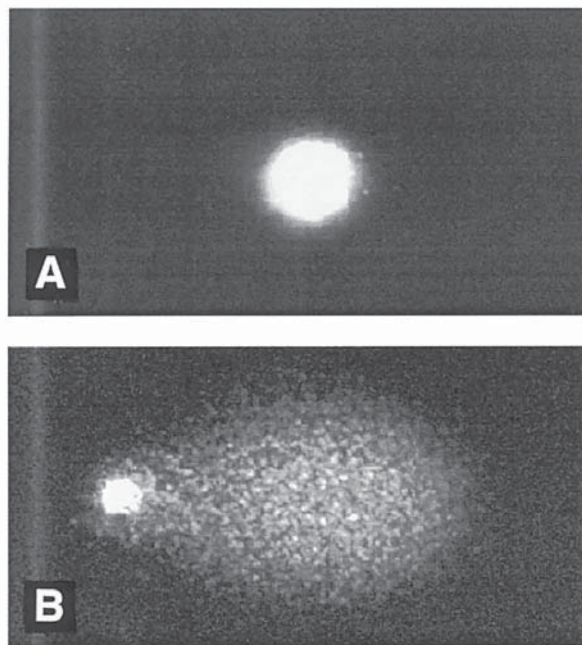


Fig. 1. Photomicrographs of human lymphocytes in the comet assay. (A) Untreated cell (control). (B) Cell exhibiting increased DNA migration after mutagen treatment.

The alkaline version of the comet assay was introduced by Singh and coworkers (3) in 1988. Performing electrophoresis at $\text{pH} > 13.0$, enabled the detection of DNA single-strand breaks and alkali-labile lesions. Other versions of the assay were then developed by Olive (4) which involved lysis in alkali followed by electrophoresis at either neutral or mild alkaline ($\text{pH} 12.1$) conditions to detect DNA double-strand breaks or single-strand breaks, respectively (2). Since the majority of genotoxic agents induce many more single-strand breaks and alkali-labile sites than double-strand breaks, the alkaline version ($\text{pH} > 13.0$) of the comet assay has the highest sensitivity for detecting induced DNA damage. Important improvements of the test procedure were introduced by Klaude and coworkers in 1996 (5). The use of agarose-precoated slides in combination with the drying of gels and fixation of comets led to a further simplification and a much better handling of the test.

1.1. Detection of DNA Damage

A broad spectrum of DNA-damaging agents causes increased DNA migration in the comet assay: UV and ionizing radiation, hydrogen peroxide and other radical-forming chemicals, alkylating agents, polycyclic aromatic hydro-

carbons (PAHs) and other adduct-forming compounds, radiomimetic chemicals, and various metals (**1**). In principle, the alkaline version of the comet assay detects all kinds of directly induced DNA single-strand breaks and any lesion capable of being transformed into a single-strand break at the alkaline pH used (i.e., alkali-labile sites). Crosslinks (DNA–DNA or DNA–protein), as induced by nitrogen mustard, cisplatin, cyclophosphamide, or formaldehyde, may cause problems in the standard protocol of the test, because crosslinking may stabilize chromosomal DNA and inhibit DNA migration (**6**). One way to detect crosslinking is first to induce DNA fragmentation with a reference agent (e.g., ionizing radiation or methyl methanesulfonate [MMS]) and then determine the reduced migration in the presence of the crosslinking agent (**7**). Crosslinks can also be analyzed by increasing the duration of unwinding and/or electrophoresis to such an extent that DNA from untreated control cells exhibits significant migration in contrast to DNA from treated cells, which migrates poorly (**8**). In addition to directly induced strand breakage, processes that introduce single-strand nicks in the DNA, such as incision during excision repair processes, are also detectable. In some cases (e.g., UV, PAHs), the contribution of excision repair to the induced DNA effects in the comet assay seems to be of major importance (**9**). Some specific classes of DNA base damage can be detected with the comet assay in conjunction with lesion-specific endonucleases. These enzymes, applied to the slides for a short time after lysis, nick DNA at sites of specific base alterations, and the resulting single-strand breaks can be quantified in the comet assay. Using this modification of the comet assay, oxidized DNA bases have been detected with high sensitivity with the help of endonuclease III or formamidopyrimidine-DNA-glycosylase (Fpg; *see also* Chapter 21) *in vitro* and *in vivo* (**10,11**).

1.2. Measuring DNA Repair

Probably the best general approach for the determination of DNA repair is to monitor the time-dependent removal of lesions (i.e., the decrease in DNA migration) after treatment with a DNA-damaging agent. The comet assay has been successfully used to follow the rejoining of strand breaks induced by ionizing radiation or reactive oxygen species (**3,12**) as well as the repair of various kinds of DNA damage induced by chemical mutagens (**13,14**). A useful extension of repair studies includes the use of lesion-specific enzymes, as mentioned in **Subheading 1.1.**, to follow the repair of specific types of DNA lesion. Moreover, owing to the comet assay's high sensitivity, this approach enables the analysis of very low ("physiological") levels of DNA damage (**10**). A common alternative approach is the use of repair inhibitors or repair-deficient cells. Incubation of cells with inhibitors of DNA synthesis leads to an accumulation of DNA breaks at sites of incomplete repair (**9,15**). Mutant cell lines either with a

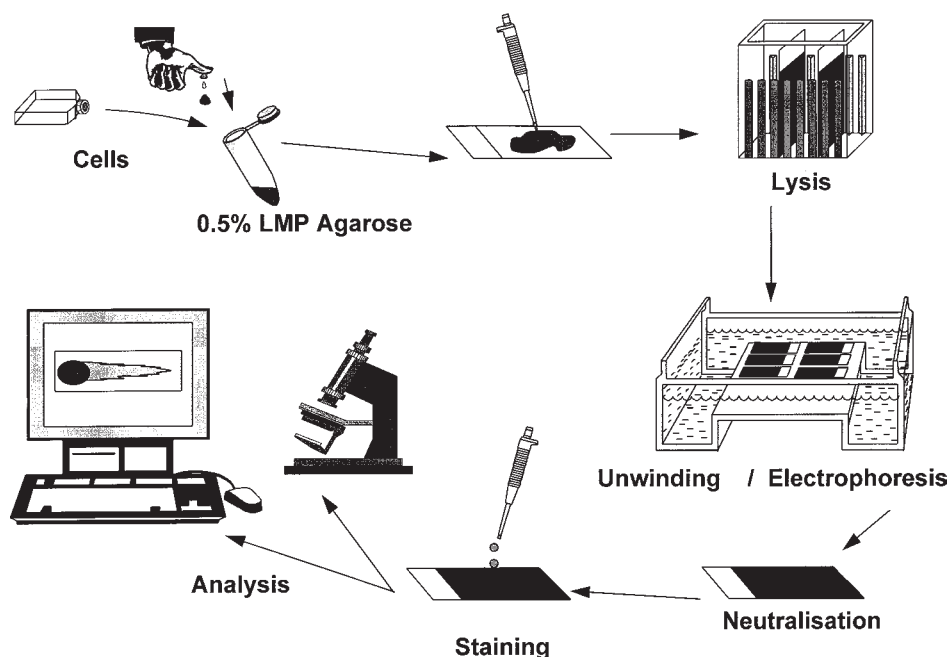


Fig. 2. Scheme for the performance of the comet assay.

specific defect in a repair pathway (e.g., xeroderma pigmentosum) or with a hypersensitivity to specific DNA-damaging agents (e.g., various mutant rodent cell lines) are well suited to elucidate the biological consequences of disturbed DNA repair or to evaluate the repair-competence of cells (9,16,17).

The purpose of this chapter is to provide information on the application of the alkaline comet assay for the investigation of DNA damage and repair in mammalian cells *in vitro*. For establishing the method, we recommend starting with experiments using blood samples and to induce DNA damage using a standard mutagen (e.g., MMS). The method described here is based on a protocol established by R. Tice according to the original work of Singh et al. (3) and includes the modifications introduced by Klaude and coworkers (5). Further modifications are described and additional cytotoxicity measurements suggested. An outline of the protocol is diagrammed in Fig. 2.

2. Materials

1. Microscope slides (with frosted end).
2. Cover slips (24 × 60 mm).
3. Normal melting-point agarose.
4. Low-melting point (LMP) agarose.

5. Horizontal gel electrophoresis unit.
6. Fluorescence microscope equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm.
7. Phosphate-buffered saline (PBS) (without Ca^{2+} and Mg^{2+}).
8. Lysing solution (1L): 2.5 M NaCl, 100 mM EDTA, 10 mM Tris (set pH to 10.0 with ~8 g solid NaOH), 1% sodium lauroyl sarcosinate. Store at room temperature. Final lysing solution (100 mL, made fresh): Add 1 mL of Triton X-100 and 10 mL of dimethyl sulfoxide (DMSO) to 89 mL of lysing solution, and then refrigerate (4°C) for 60 min before use.
9. Electrophoresis buffer: 300 mM NaOH/1 mM EDTA. Prepare from stock solutions of 10 N NaOH (200 g/500 mL of distilled H_2O), 200 mM EDTA (14.89 g/200 mL of dH_2O , pH 10.0). Store at room temperature. For 1X buffer, mix 45 mL of NaOH, 7.5 mL of EDTA, and add water to 1500 mL. Mix well. Make fresh before each run.
10. Neutralization buffer: 0.4 M Tris-HCl, pH 7.5. Store at room temperature.
11. Ethidium bromide staining solution: 10X stock: 200 $\mu\text{g/mL}$. Store at room temperature. For 1X stock, mix 1 mL with 9 mL of dH_2O and filter. **Caution:** Ethidium bromide is a mutagen. Handle it with care.

3. Methods (see Notes 1–3)

3.1. Preparation of Slides

1. Clean the slides with ethanol before use.
2. For the bottom layer, prepare 1.5% normal melting agarose (300 mg in 20 mL of PBS) and boil two to three times before use. Dip the cleaned slides briefly into the hot ($>60^\circ\text{C}$) agarose. The agarose should reach to and cover half of the frosted part of the slide to ensure that the agarose will stick properly. Wipe off the agarose from the bottom side of the slide and place the slide horizontally. This step has to be performed quickly to ensure a good distribution of the agarose. Dry the slides overnight at room temperature. Slides can be stored for several weeks.
3. Prepare 0.5% LMP agarose (100 mg in 20 mL of PBS). Microwave or heat until near boiling and the agarose dissolves. Place the LMP agarose in a 37°C water bath to cool.
4. Add 120 μL of LMP agarose (37°C) mixed with 5000–50,000 cells (*see Subheading 3.2.*) in ~5–10 μL (do not use more than 10 μL). Add a cover slip, and place the slide in a refrigerator for ~2 min (until the agarose layer hardens). Using ~10,000 cells results in ~1 cell/microscope field (250 $\times$ magnification). From this step until the end of electrophoresis, direct light irradiation should be avoided to prevent additional DNA damage.
5. Gently slip off the cover slip and slowly lower the slide into cold, freshly made lysing solution. Protect from light, and place at 4°C for a minimum of 1 h. Slides may be stored for extended periods of time in cold lysing solution (but generally not longer than 4 wk). If precipitation of the lysing solution is observed, the slides should be rinsed carefully with distilled water before electrophoresis.

3.2. Preparation of Cells (see Notes 4 and 5)

1. Whole blood: Mix ~5 μL of whole blood with 120 μL of LMP agarose, and layer onto the slide. (See **Note 6**.)
2. Isolated lymphocytes: Mix 20 μL of whole blood with 1 mL of RPMI 1640 medium in a microcentrifuge tube. Add 100 μL of Ficoll below the blood/medium mixture. Spin for 3 min at 200g. Remove 100 μL of the middle/top of the Ficoll layer, add to 1 mL of medium, and mix. Spin for 3 min to pellet the lymphocytes. Pour off the supernatant, resuspend the pellet in 120 μL of LMP agarose, and layer onto the slide.
3. Cell cultures:
 - a. Monolayer cultures: Gently trypsinize the cells (for ~2 min with 0.15% trypsin, stop by adding serum or complete cell culture medium) to yield approx 1×10^6 cells/mL. Add 5 μL of cell suspension to 120 μL of LMP agarose, and layer onto the slide.
 - b. Suspension cultures: Add ~10,000 cells in 10 μL (or smaller volume) to 120 μL of LMP agarose and layer onto the slide.

3.3. Electrophoresis and Staining

1. After at least 1 h at 4°C, gently remove the slides from the lysing solution. (See **Note 7**.)
2. Place the slides in the gel box near the anode (+) end, positioning them as close together as possible. Fill in any gaps with blank slides.
3. Fill the buffer reservoirs with freshly made electrophoresis buffer (4°C) until the slides are completely covered (avoid bubbles over the agarose). Perform the electrophoresis in an ice bath (4°C).
4. Let the slides sit in the alkaline buffer for 20–60 min to allow unwinding of the DNA and the expression of alkali-labile damage. For most experiments with cultured cells, 20 min are recommended.
5. Turn on the power supply to 25 V (~0.8–1.5 V/cm, depending on gel box size), and adjust the current to 300 mA by slowly raising or lowering the buffer level. Depending on the purpose of the study and on the extent of migration in the control samples, allow the electrophoresis to run for 20–40 min. For most experiments, 20 min are recommended.
6. Turn off the power. Gently lift the slides from the buffer, and place on a staining tray. Coat the slides with drops of neutralization buffer, and let sit for at least 5 min. Repeat two more times.
7. Drain the slides, rinse carefully with distilled water, and let them dry (inclined) at room temperature. Slides can be stored for a longer time before staining. To stain, rinse the slides briefly in distilled water, add 30 μL of 1X ethidium bromide staining solution, and cover with a cover slip.

Slides should be stained one by one and evaluated immediately. It is possible to rinse stained (evaluated) slides in distilled water, remove the coverslip, let the slides dry, and stain them at a later time for re-evaluation.

3.4. Evaluation of DNA Effects

For visualization of DNA damage, observations are made of ethidium bromide-stained DNA at 250× (or 400×) magnification using a fluorescence microscope. Generally, 50 randomly selected cells/sample are analyzed. In principle, evaluation can be done in four different ways:

1. The percentage of cells with tail vs those without is determined.
2. Cells are scored visually according to tail size into five classes (from undamaged, 0, to maximally damaged, 4). Thus, the total score for 50 comets can range from 0 (all undamaged) to 200 (all maximally damaged) (12).
3. Cells are analyzed using a calibrated scale in the ocular lens of the microscope. For each cell, the image length (diameter of the nucleus plus migrated DNA) is measured in microns, and the mean is calculated.
4. An image analysis system linked to a gated CCD camera is used to quantitate DNA image length, head length, tail length, and tail intensity. The statistical variants usually used include DNA migration (image length, tail length), tail intensity, and tail moment. It should be noted that the calculation of tail moment (DNA migration × tail intensity) in different image analysis systems may not be based on the same parameters.

For the statistical analysis of comet assay data, a variety of parametric and nonparametric statistical methods are used. The most appropriate means of statistical analysis depends on the kind of study and has to take into account the various sources of assay variability. For a powerful statistical analysis of *in vitro* test data, appropriate replication and repeat experiments have to be performed. When migration length is used as the measure of DNA damage, the median of the 50 cells/experimental point and the mean from repeat experiments should be determined. Mean migration should not be used, since a normal size distribution is not observed. Analyses are mainly based on changes in group mean response, but attention should also be paid to the distribution among cells, which often provides additional important information.

4. Notes

1. Many technical variations have been used including changes in the concentration and amount of LMP agarose, the composition of the lysing solution and the lysis time, the alkaline unwinding, the electrophoresis buffer and electrophoretic conditions, DNA-specific dyes for staining, and so forth (for details, *see 1*). Some of these variables may affect the sensitivity of the test. To allow for a comparison obtained in different laboratories and for a critical evaluation of data, it is absolutely necessary to describe clearly the technical details of the method employed.
2. Although the protocol described here detects a broad spectrum of DNA-damaging agents with high sensitivity, modifications have been suggested that can further increase the sensitivity and may be advantageous for certain applications (18,19). These modifications include the addition of radical scavengers to the

- electrophoresis buffer (to reduce damage during prolonged electrophoresis), the addition of proteinase K to the lysing solution (to remove residual proteins that might inhibit DNA migration), and the use of the DNA dye YOYO-1 (Molecular Probes, Eugene, OR) (to increase the sensitivity for the detection of migrated DNA).
3. It is strongly recommended to include some measure of cytotoxicity in any study. Acute lethal effects can easily be determined by fluorochrome-mediated viability tests. However, since cell survival may be significantly reduced in the absence of acute cytotoxicity, tests indicating long-term survivability (e.g., plating efficiency) should also be considered (20). The comet assay has not yet been sufficiently validated and may be sensitive to nongenotoxic cell killing. Data suggest that apoptotic or necrotic cells show a certain microscopic image, i.e., comets with no heads and nearly all DNA in the tail. For the evaluation of genotoxic effects, it is recommended to record these cells, but to exclude them from evaluation under the principle that they represent dead cells.
 4. Many cell types have been tried, and it is a strength of the comet assay that virtually any eukaryote cell population is amenable to analysis. The comet assay is particularly suited for the investigation of organ- or tissue-specific genotoxic effects in vivo (for a review, *see 1*), the only requirement being the preparation of an intact single-cell suspension.
 5. For in vitro tests, cells are usually incubated with the test substance for a defined period of time (*see Note 6*), then mixed with LMP agarose, and added to the slide. A modified protocol that may be performed in combination with the standard comet assay recommends the treatment after lysis. Under these conditions, the lysed cells are no longer held under the regulation of any metabolic pathway or membrane barrier (21).
 6. For the demonstration of a positive effect, mix 200 μ L of heparinized whole blood with 50 μ L of a 0.25 mM MMS (final concentration: 0.05 mM), incubate for 1 h at 37°C, and then use 10 μ L for the test.
 7. If specific types of base damage are to be analyzed by using lesion-specific endonucleases, the standard protocol has to be modified in the following way: After at least 1 h at 4°C, gently remove the slides from the lysing solution and wash three times in enzyme buffer. Drain the slides, and cover with 200 μ L of buffer or enzyme in buffer. Seal with a cover slip and incubate for 30 min at 37°C. Remove the cover slip, rinse slides with PBS, and place them on the electrophoresis box (10,11).

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Measuring the Formation and Repair of UV Photoproducts by Ligation-Mediated PCR

Gerd P. Pfeifer and Reinhard Dammann

1. Introduction

Several types of DNA lesions are formed on irradiation of cells with ultraviolet (UV) light (1,2). The two most frequent lesions are the cyclobutane pyrimidine dimers (CPDs) and the pyrimidine (6-4) pyrimidone photoproducts ([6-4] photoproducts; [6-4]PPs). In addition, UV irradiation produces, although at significantly lower levels, purine dimers, a photoproduct at TpA sequences, and pyrimidine monoadducts, such as photohydrates (3). CPDs are formed between the 5,6 bonds of two adjacent pyrimidines. The (6-4)PPs are characterized by covalent bonds between positions 6 and 4 of two adjacent pyrimidines and arise through a rearrangement mechanism. CPDs are about three times more frequent than (6-4)PPs (4). Both photoproducts are mutagenic, but it is believed that the CPD is the more harmful lesion in mammalian cells (2,5). CPDs persist much longer in mammalian DNA than (6-4)PPs owing to a significantly faster repair of (6-4)PPs (6). Perhaps because of the inefficient recognition of CPDs by the general nucleotide excision repair (NER) pathway, cells have developed other means to cope with this lesion. CPDs are subject to a specialized transcription-coupled repair pathway (7), which removes these lesions selectively from the template strand of genes transcribed by RNA polymerase II (8), but not from genes transcribed by RNA polymerase I (9-12) or RNA polymerase III (13).

NER plays an important role in preventing UV-induced mutagenesis and carcinogenesis. Several human genetic disorders are characterized by a defect in DNA repair. Cells from patients suffering from xeroderma pigmentosum (XP) or Cockayne syndrome (CS) are hypersensitive to UV light (14-16). XP is a genetic disease characterized by seven different functional complementa-

tion groups. The incidence of skin cancer in certain XP patients is increased by several thousand-fold relative to the normal population (17), and this probably is a consequence of a severe deficiency in DNA repair of UV photolesions.

In our own previous work, we have developed a technique based on ligation-mediated PCR (LMPCR), which can be used to analyze the distribution and repair of UV photoproducts along specific human genes at the DNA sequence level (13,18–24). LMPCR methods for the detection of (6-4)PPs (18) and CPDs (19) are available. LMPCR provides a sufficient level of sensitivity even when rather low UV doses (10–20 J/m² of UV-C) are used, and the repair of CPDs can be measured reliably at these doses (22–25). Since (6-4)PPs are less frequent than CPDs and the detection method produces a higher background in nonirradiated DNA, the repair of this lesion has not yet been analyzed by LMPCR.

The ability of LMPCR to detect DNA adducts depends on the specific conversion of the adduct into a strand break with a 5'-phosphate group. (6-4)PPs and their Dewar isomers can be converted by heating UV-irradiated DNA in piperidine (26). CPDs are alkali-resistant, but can be mapped at the DNA sequence level by cleavage with specific enzymes, such as T4 endonuclease V (27). T4 endonuclease V cleaves the glycosidic bond of the 5'-base in a pyrimidine dimer and also cleaves the sugar phosphate backbone between the two dimerized pyrimidines. The digestion products still contain a dimerized pyrimidine base at the cleavage site. We found that these fragments could be amplified efficiently by LMPCR only after photoreversal of the cyclobutane ring of the dimerized base with *Escherichia coli* photolyase to result in a normal base on a 5'-terminal sugar-phosphate (19). **Figure 1** shows how UV photoproducts are converted into DNA strand breaks.

The LMPCR technique is based on the ligation of an oligonucleotide linker onto the 5'-end of each DNA molecule that was created by the strand cleavage reactions. This ligation provides a common sequence on all 5'-ends allowing exponential PCR to be used for signal amplification. Thus by taking advantage of the specificity and sensitivity of PCR, one needs only a microgram of mammalian DNA per lane to obtain good-quality DNA sequence ladders. The general LMPCR procedure is outlined in **Fig. 2**. The first step of the procedure is cleavage of DNA, generating molecules with a 5'-phosphate group by converting UV photolesions into strand breaks. Next, primer extension of a gene-specific oligonucleotide (primer 1) generates molecules that have a blunt end on one side. Linkers are ligated to these blunt ends, and then an exponential PCR amplification of the linker-ligated fragments is done using the longer oligonucleotide of the linker (linker-primer) and a second gene-specific primer (primer 2). After 18–20 PCR amplification cycles, the DNA fragments are separated on sequencing gels, electroblotted onto nylon membranes, and

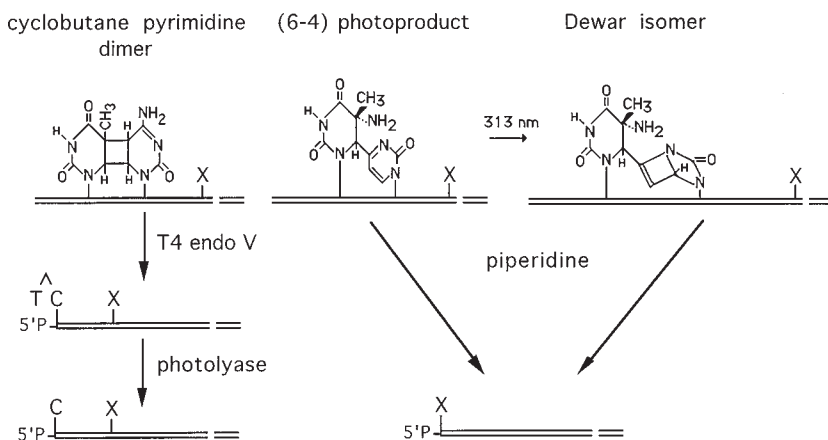


Fig. 1. Detection of cyclobutane pyrimidine dimers and (6-4) photoproducts at the trinucleotide sequence "T-C-X". CPDs are converted into DNA strand breaks with a 5'-phosphate group by cleavage with T4 endonuclease V and by photolyase treatment to create ligatable ends. The resulting DNA break positions can be detected by ligation-mediated PCR. (6-4)PPs and their Dewar isomers are converted into DNA strand breaks with 5'-phosphate groups by alkaline cleavage. Note that an amplification product derived from a (6-4)PP is one nucleotide shorter than the product derived from a CPD at the same dipyrimidine sequence. Only one strand of the DNA duplex is shown.

hybridized with a gene-specific probe to visualize the sequence ladders. The arrangement of primers in a typical LMPCR primer set is illustrated in **Fig. 3**. In this chapter, we provide detailed protocols for analysis of UV photoproducts and their repair rates by ligation-mediated PCR.

2. Materials

2.1. Irradiation of Cells

1. UV light source: Light sources emitting 254 nm light are available in most laboratories as germicidal lamps or UV crosslinking devices. UV-B irradiation can be performed with UV-B lamps such as a Philips TL 20W/12RS lamp.
2. UVX radiometer (Ultraviolet Products, San Gabriel, CA).

2.2. DNA Isolation

1. Buffer A: 0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 60 mM Tris-HCl, pH 8.0, 0.5 mM spermidine, 0.15 mM spermine, 2 mM EDTA.
2. Nonidet P40.
3. Buffer B: 150 mM NaCl, 5 mM EDTA, pH 8.0.
4. Buffer C: 20 mM Tris-HCl, pH 8.0, 20 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate (SDS).

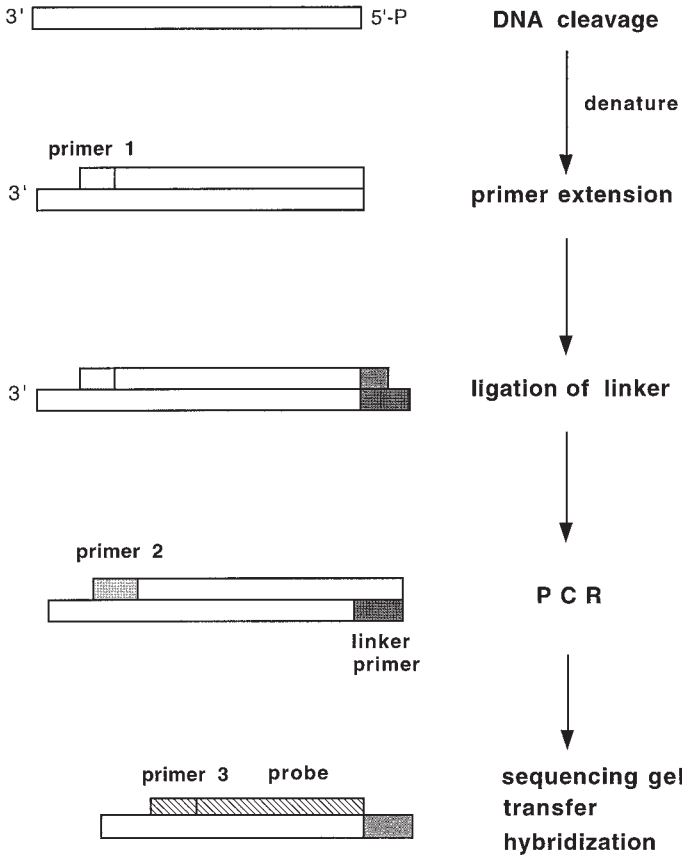


Fig. 2. Outline of the ligation-mediated PCR procedure. The steps include cleavage and denaturation of genomic DNA, annealing and extension of primer 1, ligation of the linker, PCR amplification of gene-specific fragments with primer 2 and the linker-primer, detection of the sequence ladder by gel electrophoresis, electroblotting, and hybridization with a single-stranded probe made with primer 3.

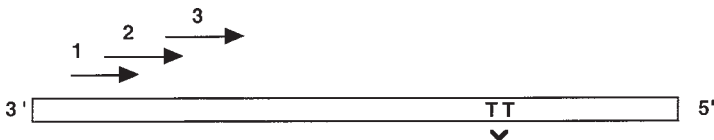


Fig. 3. Arrangement of primers in an LMPCR primer set to analyze UV photo-products on the lower (transcribed) DNA strand of a gene. Primer 1 is used for linear primer extension before ligation, primer 2 is used for PCR, and primer 3 is used to make a single-stranded hybridization probe from a cloned template.

5. Proteinase K.
6. DNase-free RNase A.
7. Phenol, equilibrated with 0.1 M Tris-HCl, pH 8.0.
8. Chloroform.
9. Ethanol.
10. 3 M sodium acetate, pH 5.2.
11. TE buffer: 10 mM Tris-HCl, pH 7.6, 1 mM EDTA.

2.3. Cleavage of DNA at Sites of UV Photodamage

1. Piperidine (Fluka, Buchs, Switzerland), 1 M, freshly prepared.
2. 10X T4 endonuclease V buffer: 500 mM Tris-HCl, pH 7.6, 500 mM NaCl, 10 mM EDTA, 10 mM dithiothreitol (DTT), 1 mg/mL bovine serum albumin (BSA).
3. T4 endonuclease V: This enzyme was kindly provided by R. S. Lloyd, University of Texas; it is also commercially available from Epicentre Technologies (Madison, WI), or from Texagen (Plano, TX).
4. *E. coli* photolyase: This enzyme was kindly provided by A. Sancar (University of North Carolina at Chapel Hill).
5. Two 360-nm black lights (Sylvania 15W F15T8).

2.4. Estimation of Cleavage Frequency by Alkaline Agarose Gels

1. Agarose.
2. 50 mM NaCl, 4 mM EDTA.
3. Running buffer: 30 mM NaOH, 2 mM EDTA.
4. Loading dye: 50% glycerol, 1 M NaOH, 0.05% bromocresol green.
5. 0.1 M Tris-HCl, pH 7.5.
6. 1 µg/mL Ethidium bromide.

2.5. Ligation-Mediated PCR

1. Oligonucleotide primers for primer extension. The primers used as primer 1 (Sequenase primers) are 15- to 20-mer with a calculated T_m of 48–56°C (*see Note 1*). Primers are prepared as stock solutions of 50 pmol/µL in water or TE buffer and are kept at –20°C.
2. 5X Sequenase buffer: 250 mM NaCl, 200 mM Tris-HCl, pH 7.7.
3. Mg-DTT-dNTP mix: 20 mM MgCl₂, 20 mM DTT, 0.25 mM of each dNTP.
4. Sequenase 2.0 (United States Biochemical), 13 U/µL.
5. 300 mM Tris-HCl, pH 7.7.
6. 2 M Tris-HCl, pH 7.7.
7. Linker: The double-stranded linker is prepared in 250 mM Tris-HCl, pH 7.7, by annealing a 25-mer (5'-GCGGTGACCCGGGAGATCTGAATTC, 20 pmol/µL) to an 11-mer (5'-GAATTCAGATC, 20 pmol/µL) by heating to 95°C for 3 min and gradually cooling to 4°C over a time period of 3 h. Linkers can be stored at –20°C for at least 3 mo. They are thawed and kept on ice.

8. Ligation mix (per reaction): 13.33 mM MgCl₂, 30 mM DTT, 1.66 mM ATP, 83 µg/mL BSA, 3 U T4 DNA ligase (Promega), and 100 pmol of linker (=5 µL linker).
9. *E. coli* tRNA.
10. 2X *Taq* polymerase mix: 20 mM Tris-HCl, pH 8.9, 80 mM NaCl, 0.02% gelatin, 4 mM MgCl₂, and dNTPs at 0.4 mM each.
11. Oligonucleotide primers for PCR: The primers used in the amplification step (primer 2) are 20- to 30-mers with a calculated T_m between 60 and 68°C (see **Note 2**); 10 pmol of the gene-specific primer (primer 2) and 10 pmol of the 25-mer linker-primer (5'-GCGGTGACCCGGGAGATCTGAATTC) are used per reaction along with 3 U of *Taq* polymerase, and these components can be included in the 2X *Taq* polymerase mix.
12. *Taq* polymerase.
13. Mineral oil.
14. 400 mM EDTA, pH 7.7.

2.6. Sequencing Gel Analysis of Reaction Products

1. Formamide loading buffer: 94% formamide, 2 mM EDTA pH 7.7, 0.05% xylene cyanol, 0.05% bromophenol blue.
2. 1 M TBE: 1 M Tris, 0.83 M boric acid, 10 mM EDTA, pH ~8.3.
3. Whatman 3MM and Whatman 17 paper (Whatman, Clifton, NJ).
4. Gene Screen nylon membranes (New England Nuclear, Boston, MA).
5. Electrophoresis apparatus (Owl Scientific, Cambridge, MA) and high-amperage power supply.
6. An appropriate plasmid or PCR product containing the sequences of interest.
7. Oligonucleotide primer to make the hybridization probe: This primer is used together with the cloned template and *Taq* polymerase to make single-stranded hybridization probes (see **Note 3**).
8. [³²P]dCTP (3000 Ci/mmol).
9. Ammonium acetate, 7.5 M.
10. Hybridization buffer: 0.25 M sodium phosphate, pH 7.2, 1 mM EDTA, 7% SDS, 1% BSA.
11. Washing buffer: 20 mM sodium phosphate, pH 7.2, 1 mM EDTA, 1% SDS.
12. Kodak XAR-5 film.

2.7. Data Analysis

Use Molecular Dynamics scanner (Sunnyvale, CA) and ImageQuant™ software.

3. Methods

3.1. Irradiation of Cells

Approximately 2–5 × 10⁶ cells are typically used for irradiation. Cells that grow as monolayers in Petri dishes, such as fibroblasts or keratinocytes, are

irradiated with a germicidal (UV-C) lamp after removal of the medium and washing with phosphate-buffered saline (PBS). It is also possible to use a UV-B irradiation source (*see Note 4*). UV doses are measured with a UV radio-meter. Typical UV doses for DNA repair assays of CPDs are 10–20 J/m² of 254 nm light (*see Note 5*).

3.2. DNA Isolation

1. Lyse the cells after UV irradiation by adding to the plate 10 mL of buffer A containing 0.5% Nonidet P40. This step will release nuclei and removes most of the cytoplasmic RNA. Transfer the suspension to a 50-mL tube. Incubate on ice for 5 min.
2. Centrifuge at 1000g for 5 min at 4°C.
3. Wash the nuclear pellet once with 15 mL of buffer A.
4. Resuspend the nuclei thoroughly in 2–5 mL of buffer B, and add 1 vol of buffer C, containing 600 µg/mL of proteinase K (added just before use). Incubate for 1 h at 37°C.
5. Add DNase-free RNase A to a final concentration of 100 µg/mL. Incubate for 30 min at 37°C (*see Note 6*).
6. Extract with one vol of buffer-saturated phenol. Then, extract with 0.5 vol of phenol and 0.5 vol of chloroform. Repeat this step until the aqueous phase is clear and no interface remains. Finally, extract with 1 vol of chloroform.
7. Add 0.1 vol of 3 M sodium acetate, pH 5.2, and precipitate the DNA with 2.5 vol of ethanol at room temperature.
8. Centrifuge at 2000g for 1 min (*see Note 7*). Wash the pellet with 75% ethanol and air-dry briefly.
9. Dissolve the DNA in TE buffer to a concentration of approx 0.2 µg/µL. Keep at 4°C overnight. The DNA should be well dissolved before cleavage with T4 endonuclease V.

3.3. Cleavage of DNA at Sites of UV Photodamage

3.3.1. (6-4) Photoproducts

To obtain DNA fragments with a 5'-phosphate group at the positions of (6-4)PPs, the DNA is heated in 1 M piperidine. This will destroy the photolesion and create strand breaks with 5'-phosphate groups, since the sugar residue at the 3'-base of the (6-4)PP is destroyed by β -elimination.

1. Dissolve 10–50 µg of UV-irradiated DNA in 100 µL of 1 M piperidine.
2. Heat the DNA at 90°C for 30 min in a heating block (use lid locks to prevent the tubes from popping). Cool the samples briefly on ice after heating.
3. Add 10 µL of 3 M sodium acetate pH 5.2 and 2.5 vol of ethanol. Put on dry ice for 20 min.
4. Spin at 14,000 rpm (~15,800g) in an Eppendorf centrifuge for 15 min.
5. Wash twice with 1 mL of 75% ethanol.

6. Remove traces of remaining piperidine by drying the sample overnight in a vacuum concentrator. Dissolve the DNA in TE buffer to a concentration of approximately 0.5–1 $\mu\text{g}/\mu\text{L}$.
7. Determine the frequency of (6–4)PPs by separating 1 μg of the DNA on a 1.5% alkaline agarose gel (*see Subheading 3.4.*).

3.3.2. Cyclobutane Pyrimidine Dimers

The DNA is first incubated with T4 endonuclease V and then with *E. coli* photolyase (*see Fig. 1*) to create fragments with 5'-phosphate groups and ligatable ends.

1. Mix the UV-irradiated DNA (~10 μg in 50 μL) with 10 μL of 10X T4 endonuclease V buffer and a saturating amount of T4 endonuclease V in a final volume of 100 μL . Saturating amounts of T4 endonuclease V activity can be determined by incubating UV-irradiated (20 J/m²) genomic DNA with various enzyme dilutions and separating the cleavage products on alkaline agarose gels (*see Subheading 3.4.*). Incubate at 37°C for 1 h.
2. Add DTT to a final concentration of 10 mM. Add 5 μg of *E. coli* photolyase under yellow light.
3. Irradiate the samples in 1.5-mL tubes from two 360-nm black lights filtered through 0.5-cm thick window glass for 1 h at room temperature at a distance of 3 cm.
4. Extract once with phenol-chloroform.
5. Precipitate the DNA by adding 1/10 vol of 3 M sodium acetate, pH 5.2 and 2.5 vol of ethanol. Leave on dry ice for 20 min. Centrifuge the samples for 10 min at 14,000 (~15,800g) rpm at 4°C.
6. Wash the pellets with 1 mL of 75% ethanol and air-dry.
7. Dissolve the DNA in TE buffer to a concentration of about 0.5–1 $\mu\text{g}/\mu\text{L}$.
8. Determine the frequency of CPDs by running 1 μg of the samples on a 1.5% alkaline agarose gel.

3.4. Estimation of Cleavage Frequency by Alkaline Agarose Gels

The approximate size of the fragments obtained after cleavage of UV-irradiated DNA is determined on an alkaline 1.5% agarose gel.

1. Prepare a 1.5% agarose gel by dissolving agarose in 50 mM NaCl, 4 mM EDTA and microwaving. Pour the gel.
2. After the gel solidifies, soak it in alkaline running buffer for at least 2 h.
3. Dilute the DNA sample with 1 vol of loading dye. Incubate for 15 min at room temperature. Load the samples.
4. Run the gel at 40 V for 3–4 h.
5. Neutralize the gel by soaking for 60 min in 500 mL of 0.1 M Tris-HCl, pH 7.5.
6. Stain with 1 $\mu\text{g}/\text{mL}$ ethidium bromide for 30 min.
7. Destain in water for 30 min.

See **ref. 24** for examples of UV-irradiated DNA analyzed by alkaline agarose gel electrophoresis.

3.5. Ligation-Mediated PCR

1. Mix in a siliconized 1.5 mL tube: 1–2 μg of cleaved DNA (*see Note 8*), 0.6 pmol of primer 1, and 3 μL of 5X Sequenase buffer in a final volume of 15 μL .
2. Incubate at 95°C for 3 min, and then at 45°C for 30 min.
3. Cool on ice, and then centrifuge for 5 s.
4. Add 7.5 μL of cold, freshly prepared Mg-DTT-dNTP mix.
5. Add 1.5 μL of Sequenase, diluted 1:4 in cold 10 mM Tris-HCl (pH 7.7).
6. Incubate at 48°C for 15 min, and then cool on ice.
7. Add 6 μL 300 mM Tris-HCl (pH 7.7).
8. Incubate at 67°C for 15 min (to inactivate the Sequenase).
9. Cool on ice, and centrifuge for 5 s.
10. Add 45 μL of freshly prepared ligation mix.
11. Incubate overnight at 18°C.
12. Incubate for 10 min at 70°C (to inactivate the DNA ligase).
13. Add 8.4 μL of 3 M sodium acetate (pH 5.2), 10 μg of *E. coli* tRNA, and 220 μL of ethanol.
14. Put the samples on dry ice for 20 min.
15. Centrifuge for 15 min at 4°C in an Eppendorf centrifuge.
16. Wash the pellets with 950 μL of 75% ethanol.
17. Remove the ethanol residue in a Speed Vac or by air-drying.
18. Dissolve the pellets in 50- μL of H₂O and transfer to 0.5-mL siliconized tubes.
19. Add 50 μL of freshly prepared 2X *Taq* polymerase mix containing the primers and enzyme, and mix by pipeting.
20. Cover the samples with 50 μL of mineral oil and spin briefly.
21. Cycle 18–20 times at 95°C for 1 min, 60–66°C for 2 min, and 76°C for 3 min. The temperature during the annealing step is at the calculated T_m of the gene-specific primer.
22. To extend completely all DNA fragments and add an extra nucleotide through *Taq* polymerase's terminal transferase activity, an additional *Taq* polymerase step is performed (*see Note 9*). Add 1 U of *Taq* polymerase/sample together with 10 μL of reaction buffer. Incubate for 10 min at 74°C.
23. Stop the reaction by adding sodium acetate to 300 mM, EDTA to 10 mM, and add 10 μg of tRNA.
24. Extract with 70 μL of phenol and 120 μL of chloroform (premixed).
25. Add 2.5 vol of ethanol, and put on dry ice for 20 min.
26. Centrifuge the samples for 15 min in an Eppendorf centrifuge at 4°C.
27. Wash the pellets in 1 mL of 75% ethanol.
28. Dry the pellets in a vacuum concentrator.

3.6. Sequencing Gel Analysis of Reaction Products

1. Dissolve the pellets in 1.5 μL of water, and add 3 μL of formamide loading buffer.

2. Heat the samples to 95°C for 2 min prior to loading. Loading is performed with a very thin flat tip. Load only one-half of the samples or less. The gel is 0.4 mm thick and 60 cm long, consisting of 8% polyacrylamide (the ratio of acrylamide to *bis*-acrylamide is 29:1) and 7 M urea in 0.1 M TBE. To allow identification of the sequence position of the UV-specific bands, include Maxam-Gilbert sequencing standards prepared from genomic DNA as previously described (28).
3. Run the gel until the xylene cyanol marker reaches the bottom. Fragments below the xylene cyanol dye do not hybridize significantly.
4. After the run, transfer the gel (i.e., the bottom 40 cm of it) to Whatman 3MM paper, and cover with Saran Wrap.
5. Electroblotting of the gel piece can be performed with a simple homemade apparatus (28) or with a transfer box available from Owl Scientific (*see Note 10*). Pile three layers of Whatman 17 paper, 43 × 19 cm², presoaked in 90 mM TBE, onto the lower electrode. Squeeze the paper with a roller to remove air bubbles between the paper layers. Place the gel piece covered with Saran Wrap onto the paper, and remove the air bubbles between the gel and the paper by wiping over the Saran wrap with a soft tissue. Remove the Saran Wrap, and cover the gel with a GeneScreen nylon membrane cut somewhat larger than the gel and presoaked in 90 mM TBE. Put three layers of presoaked Whatman 17 paper onto the nylon membrane, carefully removing trapped air with a roller. Place the upper electrode onto the paper. Perform the electroblotting procedure at 1.6 A. After 30 min, remove the nylon membrane and mark the DNA side. A high-amperage power supply is required for this transfer.
6. After electroblotting, dry the membrane briefly at room temperature, and then crosslink the DNA by UV irradiation. UV irradiation is performed in a commercially available crosslinker or by mounting six 254-nm germicidal UV tubes (15 W) into an inverted transilluminator from which the upper lid has been removed. With this device, the distance between membrane and UV bulbs is 20 cm; the irradiation time is 30 s.
7. Perform the hybridization in rotating 250-mL plastic or glass cylinders in a hybridization oven. Soak the nylon membranes briefly in 90 mM TBE. Roll them into the cylinders by unspooling from a thick glass rod so that the membranes stick completely to the walls of the cylinders without air pockets. Prehybridize with 15 mL of hybridization buffer for 10 min. For hybridization, dilute the labeled probe into 7 mL of hybridization buffer. Both the prehybridization and hybridization are performed at 62°C.
8. To prepare labeled single-stranded probes, 200–300 nt in length, use repeated primer extension by *Taq* polymerase with a single primer (primer 3) on a double-stranded template DNA (20). This can be either plasmid DNA restriction-cut approx 200–300 nt 3' to the binding site of primer 3 or a PCR product containing the target area of interest. To prepare the single-stranded probe, mix 50 ng of the respective restriction-cut plasmid DNA (or 10 ng of the gel-purified PCR product) with primer 3 (20 pmol), 100 µCi of [³²P]dCTP, 10 µM of the other three dNTPs, 10 mM Tris-HCl, pH 8.9, 40 mM NaCl, 0.01% gelatin, 2 mM MgCl₂, and 3 U of

Taq polymerase in a volume of 100 μ L. Perform 35 cycles at 95°C for 1 min, 60–66°C for 1 min, and 75°C for 2 min. Recover the probe by phenol/chloroform extraction, addition of ammonium acetate to a concentration of 0.7 *M*, ethanol precipitation at room temperature, and centrifugation.

9. After hybridization, wash each nylon membrane with 2 L of washing buffer at 60°C. Perform several washing steps in a dish at room temperature with prewarmed buffer. After washing, dry the membranes briefly at room temperature, wrap in Saran wrap, and expose to Kodak XAR-5 films. If the procedure has been done without error, a result can be seen after 0.5–8 h of exposure with two intensifying screens at –80°C. Nylon membranes can be used for rehybridization (29). Probes can be stripped from the nylon membranes by soaking in 0.2 *M* NaOH for 30 min at 45°C.

3.7. Data Analysis

Data analysis (*see Note 11*) is routinely performed by phosphorimager analysis using a Molecular Dynamics scanner. For quantitation of repair rates, nylon membranes are exposed to the phosphorimager, and radioactivity is determined in all CPD-specific bands of the sequencing gel that show a consistent and measurable signal above background. Background values (from the control lanes without UV irradiation) are subtracted. A repair curve can be established for each CPD position that gives a sufficient signal above background. The time at which 50% of the initial damage is removed can then be determined from this curve (*see refs. 13 and 23*, for examples).

4. Notes

1. Calculation of the T_m is done with the Oligo™ computer program in the DNA amplification mode (30). Primers do not need to be gel-purified, if the oligonucleotide synthesis quality is sufficiently good (<5% of n-1 material on analytical polyacrylamide gels). If a specific target area is to be analyzed (e.g., a defined sequence position), primer 1 should be located approx 100 nt upstream of this target.
2. Primer 2 is designed to extend 3' to primer 1. Primer 2 can overlap several bases with primer 1, but we have also had good results with a second primer that overlapped only one or two bases with the first.
3. The primer that is used to make the single-stranded probe (primer 3) should be on the same strand just 3' to the amplification primer (primer 2) and should have a T_m of 60–68°C (*see Fig. 3*). It should not overlap more than 8–10 bases with primer 2.
4. UV-B light emitted from sun lamps sufficiently penetrates the plastic material of Petri dishes and can be administered from the bottom of the dish without the need to remove the cell-culture medium before irradiation. Polystyrene plastic dishes have a lower wavelength cutoff of 295–300 nm. When UV-B is used, a dose of about 500–1000 J/m² as measured with a 310-nm sensor is required to produce

equivalent amounts of CPD lesions as with 10–20 J/m² of UV-C.

5. At these UV doses, the average photoproduct frequencies are one CPD every 5–10 kb and one (6-4)PP every 15–30 kb.
6. Although RNase is probably active only for a very short time in the proteinase K solution, this step seems to aid in removal of traces of RNA.
7. If the initial number of cells was very low, the DNA may need to be pelleted at 10,000g for 10 min.
8. DNA concentration measurement before LMPCR is critical for DNA repair assays. To avoid wide variations in DNA concentration, the same number of cells is used as starting material for irradiation at each time-point, and care is taken that no material is lost during the DNA isolation procedure. DNA concentrations are measured by A₂₆₀ optical density reading. It is important that the DNA is completely in solution before these measurements are made.
9. If this step is omitted, double bands may occur.
10. The advantages of the hybridization approach over the end-labeling technique (31) have been discussed previously (28).
11. LMPCR can be used for quantitation of UV damage frequencies at different nucleotide positions. There are some limitations, however, owing to variations in ligation and PCR amplification of the different fragments. Fortunately, these variations are minor at sequences containing stretches of pyrimidines, which are the targets for formation of UV photoproducts. DNA repair experiments measure relative lesion frequencies over a time-course at a defined sequence position. Therefore, LMPCR amplification bias does not play a role in measuring repair rates for individual positions (32).

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PCR-Based Assays for Strand-Specific Measurement of DNA Damage and Repair I

Strand-Specific Quantitative PCR

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1. Introduction

Quantitative PCR (QPCR) has recently been adapted for use in the measurement of cellular DNA damage caused by UV irradiation and chemicals, including anticancer agents (1–3). The QPCR assay depends on the property of the lesions caused by these DNA-damaging agents to block the progress of *Taq* polymerase. Compared to Southern blotting-based methods (4), it is equally sensitive and has the advantage that it is quicker, does not require strand breaks, uses less DNA, and can be used to measure damage and repair in subgenomic (~500 bp) organizational units (3). It suffers though from one drawback which has inhibited its general use in DNA damage and repair studies: unlike Southern blotting methods, it cannot be used to measure strand-specific damage and repair. This is a serious handicap, since preferential repair of the transcribed strand in active genes (transcription-coupled repair) has been demonstrated to be an important and widespread phenomenon (5).

This and the following chapter (Chapter 20) focus on further developments of PCR carried out in our laboratory. This chapter describes a development of the QPCR method, strand-specific QPCR (ss-QPCR), which allows measurement of damage and repair on individual strands of small gene regions. Chapter 20 describes single-strand ligation PCR (slig-PCR), which can measure at the ultimate level of detection individual nucleotides in single-copy genes in mammalian cells. Both methods can be used to study damage and repair in DNA caused by a wide variety of agents, such as UV, benzopyrene, X-irradiation, aflatoxin, and so forth. The only requirement is that the lesion blocks the progression of *Taq* polymerase either physically or by causing a strand break.

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ss-QPCR allows damage to be measured in the same region of DNA as QPCR but in a strand-specific way. This is particularly important in light of recent discoveries showing heterogeneity of repair among gene regions, and that by means of transcription-coupled repair, the transcribed strand of an expressed gene can be repaired more efficiently than the non-transcribed strand (5). It is sensitive enough to be used to look at subgene functional regions, such as introns, exons, promoters, and so on. Currently, a convenient size would be between 300 and 3000 bp. However, with new reagents allowing “long-PCR” becoming available, the upper limit may be extended up to 20–30 kbp allowing ss-QPCR to be used to study entire genes.

1.1. Overview of ss-QPCR

The method is outlined in **Fig. 1** (as set up to measure lesions on the transcribed strand). DNA extracted from drug-treated and untreated cells is subjected to a first-round “linear” PCR using a single biotinylated primer (5.1tB), complementary to the transcribed strand. This PCR generates a family of single-stranded molecules, some of which will be truncated owing to the presence of a blocking lesion on the transcribed strand of the template DNA. All are captured on streptavidin-coated paramagnetic beads and washed with NaOH to remove genomic DNA, including any hybridized to the PCR products. After neutralization, the single-stranded molecules, although still attached to the beads, serve as templates in a second, exponential, PCR. In the exponential amplification, the downstream primer (primer 5.2) is complementary to the transcribed strand and is nested with respect to primer 5.1tB. The upstream primer (primer 3.2) is complementary to the nontranscribed strand, and its binding site determines the length of the gene region in which damage is to be measured. In this PCR, only those DNA molecules that were extended past the site of primer 3.2, i.e., those that were not blocked by lesions on the genomic DNA, will be exponentially amplified. Thus, provided the PCR remains in the exponential phase when stopped, the amount of product will be directly proportional to the amount of undamaged template present in the region under study of the original genomic DNA. By including a radioactive nucleotide in the exponential PCR, the extent of damage caused by particular treatments (and subsequent repair) can be accurately quantified.

2. Materials

2.1. Cell and DNA Treatment

1. Cells in suspension or monolayer culture (*see Note 1*).
2. DNA damaging agent; e.g., cisplatin, melphalan, benzopyrene, and so forth.
3. 10X Teoa (for treatment of naked DNA): 250 mM Triethanolamine, pH 7.2, 10 mM EDTA. (Store at 4°C.)
4. Drug stop solution: 0.6 M sodium acetate, pH 5.2.
5. Tissue culture plates (6-well, 24-well and/or Petri dishes).

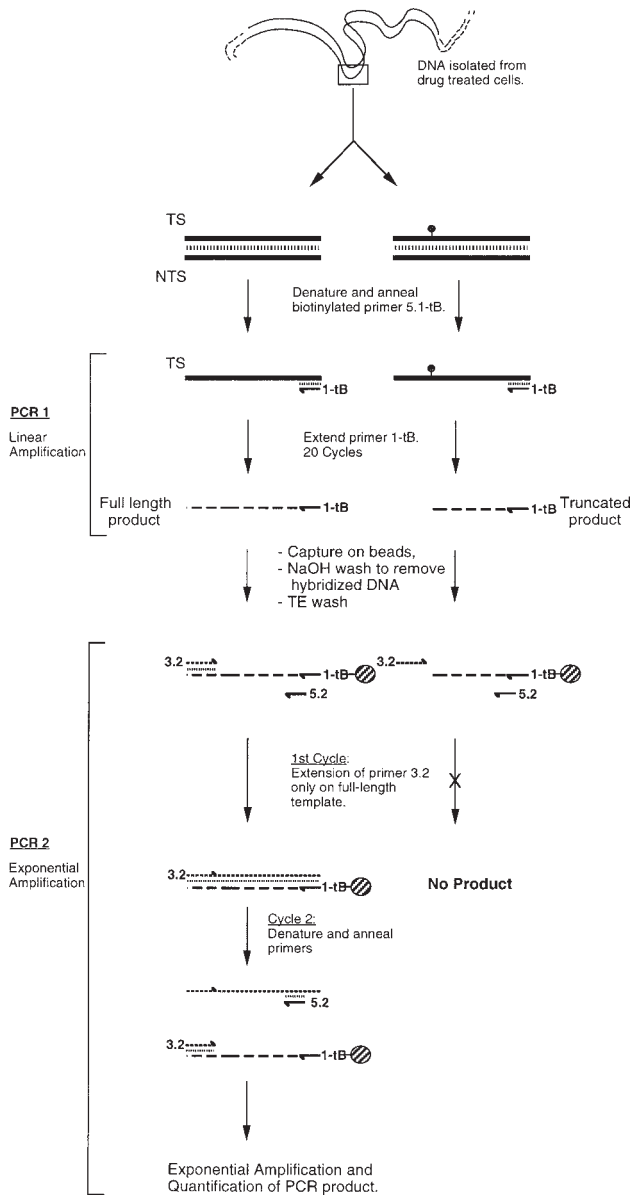


Fig. 1. ss-QPCR method (as set up to measure lesions on the transcribed strand).

2.2. DNA Isolation

1. Cell lysis buffer: 400 mM Tris-HCl, pH 8.0, 60 mM EDTA, 150 mM NaCl, 1% (w/v) sodium dodecyl sulfate (SDS). (Store at room temperature.)

2. 5 M Sodium perchlorate (store at room temperature).
3. Chloroform.
4. 37°C and 65°C water baths.
5. Rotary mixer.
6. Microcentrifuge.
7. Vacuum dryer.

2.3. Oligonucleotides

Oligonucleotides were obtained from Genosys, Pampisford, UK or MWG Biotech, Ebersberg, Germany. Store all oligonucleotides at -20°C . The oligonucleotide sequences will obviously depend on the region of the gene to be studied. Those described here can be used to study damage and repair in a 350-bp region of intron 1 of the human *N-ras* gene (*see* Appendix for sequence).

Step 1 (first-round “linear” PCR)

- a. To measure damage on the nontranscribed strand of *N-ras*: 3.1nB (5'-Biotinylated): 5'-CAG CAA GAA CCT GTT GGA AAC CAG.
- b. To measure damage on the transcribed strand of *N-ras*: 5.1tB (5' Biotinylated): 5' GGT CCT TCC ATT TGG TGC CTA CG. These primers were synthesized with biotin incorporated at the 5' end (*see* **Note 2**).

Step 2 (second-round exponential PCR)

- c. Oligo 3.2: 5'-CCA GTA ATC AGG GTT AAT TGC GAG C
- d. Oligo 5.2: 5'-ACG TGG GGA GAT CTT GGA GA

2.4. PCR

2.4.1. PCR Reagents and Equipment

1. *Taq* Polymerase (e.g., Perkin-Elmer, Beaconsfield, UK; Promega, Southampton, UK; Advanced Biotechnologies, Leatherhead, UK).
2. 10X PCR buffer (*see* **Note 3**): 200 mM $(\text{NH}_4)_2\text{SO}_4$, 750 mM Tris-HCl, pH 9.0, 0.1% (w/v) Tween.
3. 25 mM MgCl_2 (store at 4°C).
4. 10X dNTPs (Pharmacia, St. Albans, UK): make a mixture containing 2 mM each of dATP, dGTP, dCTP, and TTP; store at -20°C .
5. Thermal cycler (e.g., MJ PTC-100 with heated lid, GRI, Felsted, UK; *see* **Note 4**).
6. Mineral oil (if thermal cycler is without a heated lid facility).
7. PCR tubes—0.5 or 0.2 mL.
8. $[\alpha\text{-}^{32}\text{P}]\text{-ATP}$ 10 $\mu\text{Ci}/\mu\text{L}$ (Amersham, Aylesbury, UK).

2.4.2. Quantitation of PCR Product

Quantitation of the PCR product may be performed by one of two methods (*see* **Note 5**). One method (**Subheading 2.4.2.1.**) involves TCA precipitation of the PCR product and scintillation counting. The alternative (**Subheading**

2.4.2.2.) is quantitation by densitometric scanning of autoradiographs or phosphor image analysis after agarose-gel electrophoresis.

2.4.2.1. TCA PRECIPITATION

1. Whatman GFC filters (Whatman, Maidstone, UK; 24 mm diameter).
2. Multiple filtration manifold (Millipore, Watford, UK).
3. 5% TCA: 5% (w/v) trichloroacetic acid, 20 mM tetrasodium pyrophosphate (store at 4°C).
4. Scintillation fluid (Ecoscint, National Diagnostics, Hull, UK).
5. Scintillation counter (e.g., Beckman LS1800, Beckman, Highwycombe, UK).

2.4.2.2. AGAROSE GEL ELECTROPHORESIS AND DENSITOMETRIC SCANNING OR PHOSPHOR IMAGE ANALYSIS

1. Equipment for horizontal agarose-gel electrophoresis.
2. 50X TAE: 2 M Tris-acetate, 0.05 M EDTA (per L: 242 g Tris base, 57.1 mL of glacial acetic acid, 100 mL of 0.5 M EDTA, pH 8.0).
3. Agarose.
4. Agarose-gel loading buffer.
5. Gel dryer (suitable for agarose and acrylamide gels, e.g., Hoefer, San Francisco, CA).
6. Autoradiography cassette or phosphor image cassette.
7. Autoradiography film.
8. Standard equipment for X-ray film development.
9. Gel scanner or phosphor image analyzer.

2.4.3. Capture of Biotinylated First-Round PCR Product

1. Freshly prepared 0.4 M NaOH.
2. Streptavidin M-280 Dynabeads (Dynal, Bromborough, UK). (*See Note 2.*)
3. Magnet to capture beads. It should be able to accommodate at least six Eppendorf tubes (e.g., MPC-E6; Dynal UK).
4. 5X Washing and binding buffer (WBB): 25 mM Tris-HCl, pH 7.6, 5 mM EDTA, 5 M NaCl. (Store at 4°C.)
5. TE (pH 7.6): 10 mM Tris-HCl, pH 7.6, 1 mM EDTA. (Store at 4°C.)

3. Methods

3.1. Treatment of Isolated DNA

1. Use 0.5 µg of DNA for each ss-QPCR (i.e., per reaction).
2. Incubate the DNA with the drug for 1 h at 37°C in Teoa in a total volume of 50 µL in 1.5-mL microcentrifuge tubes.
3. Add 50 µL of 0.6 M sodium acetate “drug stop” solution, and precipitate the DNA with 3 vol of 95% ethanol.
4. Wash the DNA pellet twice with 1 mL of 75% ethanol (room temperature) and dry under vacuum.
5. Resuspend the DNA in 10 µL of deionized H₂O ready for PCR.

3.2. Treatment of Cells

3.2.1. Suspension Cultures

1. Count the cells, and resuspend at a density of 2×10^6 cells/mL in tissue-culture medium with or without fetal calf serum as required (*see Note 6*).
2. Add the required amount of drug (dissolved in tissue-culture medium or isotonic solution) to the wells of 24-well tissue culture plates.
3. Add tissue-culture medium to make the volume up to 0.5 mL.
4. Add 0.5 mL of cell suspension (1×10^6 cells), and incubate at 37°C for the appropriate time.
5. Transfer the cells to 1.5-mL microcentrifuge tubes, rinse out the wells with 0.4 mL of tissue-culture medium, and add the rinse to the tubes. Spin for 5 min at $270g$ at 4°C .
6. Remove the supernatant, and wash the cells—by resuspending and spinning—with 1-mL of tissue-culture supernatant. Repeat twice more.
7. After the washings, remove the supernatant. At this point, the cell pellet may be stored at -20°C until DNA isolation. For repair experiments, the cells are resuspended in 1 mL of tissue-culture medium supplemented with fetal calf serum, transferred to a fresh 24-well plate, and incubated at 37°C for appropriate times before harvesting the cells.

3.2.2. Adherent Cells

1. Grow cells to almost confluence in 2-cm diameter wells.
2. Treat with the drug as for suspension cells, except first mix the drug in 1 mL of tissue-culture medium to avoid adding concentrated drug directly to the cells.
3. Incubate as for suspension cells.
4. Remove the drug medium, and gently wash the cells three times with 1 mL of fresh tissue-culture medium. (*See Note 7*.)
5. If repair experiments are to be carried out, add tissue-culture medium with serum, and incubate for appropriate times.
6. Harvest the cells by trypsinization, and spin as for suspension cells. These cells may be stored at -20°C .

3.3. DNA Isolation

1. Resuspend the cell pellet in 340 μL of cell lysis buffer.
2. Add 100 μL of 5 *M* sodium perchlorate.
3. Incubate at 37°C for 20 min, mixing occasionally.
4. Transfer to a 65°C water bath, and incubate for 20 min with occasional mixing by inversion.
5. Add 580 μL of chloroform precooled to -20°C .
6. Mix by rotation for 20 min at room temperature.
7. Spin in microcentrifuge at $11,600g$ for 10 min.
8. Remove half (220 μL ; equivalent to 5×10^5 cells from suspension cultures) the upper aqueous layer, transfer to a fresh 1.5-mL microcentrifuge tube, and add 440 μL of absolute ethanol (kept at -20°C) to precipitate the DNA. (*See Note 8*.)

9. Spin at top speed in a microcentrifuge for 20 min, and wash the DNA pellet twice with 1 mL of 75% ethanol (kept at room temperature).
10. Dry the DNA pellet under vacuum.
11. Resuspend the pellet in H₂O. For ss-QPCR, resuspend in 50 μ L of H₂O, and use 10 μ L/PCR.

3.4. ss-QPCR

The efficiency and specificity of PCR often depend on the MgCl₂ concentration in addition to the annealing temperature (*see Note 9*). These parameters should therefore be established by titration before proceeding with damage experiments. Initial optimization of PCR can be performed without radioactivity, the performance being assessed by ethidium staining of agarose gels (*see Note 10*).

With ss-QPCR, DNA damage in the region of the gene under study leads to a reduction in the amount of PCR product (*see Subheading 1.*). It is essential to ensure that the only limiting component of the PCR is the template DNA and that the second-round PCR reaction remains in the exponential phase when terminated so that any damage to DNA will cause a directly proportional reduction in the amount of radioactive product. The important factors are cycle number (especially in the exponential second-round PCR) and quantity of DNA used in the first-round PCR, so preliminary experiments must be performed to determine the conditions required. First, keep the amount of DNA constant at, for example, 0.5 μ g, and vary the number of cycles (from 20 to 30) in the second-round PCR, while keeping the number of cycles in the first-round “linear” PCR fixed at 20. After quantitation of the radioactive product, the results should show an exponential increase in the amount of amplified product with increasing cycle number. A cycle number is then chosen that is well within the exponential range, but generates sufficient amplified DNA to be easily measured. In the *N-ras* example, 26 cycles were chosen (*see Note 11*). Next, perform a DNA titration using this fixed number of cycles and vary the amount of genomic DNA used in the first-round PCR (from 0.1 to 1.0 μ g). The amount of amplified product should increase linearly in direct proportion to the amount of starting DNA. These experiments thus establish the conditions under which ss-QPCR will give a quantitative measurement of the amount of DNA template available for amplification (i.e., free of damage), and DNA damage experiments can be performed.

3.4.1. Control Samples

1. Samples containing 1/3 and 2/3 the amount of (undamaged) genomic DNA are routinely used to confirm that the second-round PCR was stopped in the exponential phase. Obviously, the final values after quantitation of these samples should be 1/3 and 2/3 of the untreated DNA sample.

2. It is important to control the efficiency of the washing of the beads after the first-round PCR, since any genomic DNA carried over would serve as a template in the second PCR and be exponentially amplified leading to false results. Therefore, in the first round, PCR samples are included that contain all components except *Taq* polymerase. In the second PCR, they are treated as for the test samples, i.e., with *Taq* polymerase. Values above background with these samples would indicate genomic DNA carryover and invalidate the assay.

3.4.2. PCR-1

1. The single primer used in the first round determines on which strand the damage will be measured (i.e., using a primer complementary to the transcribed strand will detect damage on the transcribed strand). First-round “linear” PCR is carried out in a volume of 40 μL containing: 0.6 pmol of 5'-biotinylated primer 5.1tB; 1 U of *Taq* polymerase (see **Note 12**); 200 μM each of dATP, dGTP, dCTP, TTP; 2.5 mM MgCl_2 (see **Note 13**); and 10 μL of 10X PCR buffer. If necessary, add 40 μL of mineral oil overlay.
2. Place the tubes in the thermal cycler, and carry out the following program (see **Note 12**): an initial denaturation step of 3 min at 94°C and then 20 cycles of 94°C for 1 min, 60°C for 1 min (see **Note 14**), and 72°C for 1 min. This is followed by a final incubation of 4 min at 72°C.
3. If mineral oil was used, this needs to be removed as follows: Add 60 μL of H_2O and 100 μL of water-saturated chloroform. Spin the tubes briefly, remove the upper aqueous layer, and transfer to 1.5-mL microcentrifuge tubes. Add a further 100 μL of H_2O to the original PCR tubes, spin again, remove the aqueous layer, and add to the 1.5-mL microcentrifuge tubes. Precipitate the DNA with ethanol and, after drying, resuspend in 50 μL of 1X WBB.

3.4.3. Capture of PCR Products

1. Transfer streptavidin-coated Dynabeads to a 1.5-mL microcentrifuge tube. Use 5 μL /PCR plus an extra 5 μL (e.g., for 10 reactions transfer 55 μL of beads).
2. Place the tube in the magnetic rack to sediment the beads (for ~30 s) and then remove the supernatant—keep the tube in the magnetic rack!
3. Remove the tube from magnetic rack, and resuspend the beads in 200 μL of 1X WBB. Return the tube to the rack to sediment the beads; remove the supernatant. Repeat this washing process once more.
4. Resuspend the beads in 1X WBB using 40 μL /PCR (i.e., for 10 tubes resuspend in 400 μL). Mix well and transfer 40- μL aliquots to 1.5-mL microcentrifuge tubes.
5. Place the tubes in a rack to capture the beads, and remove the supernatant. The beads are now ready for the addition of the PCR mix.
6. To the 40- μL PCR mix add 10 μL of 5X WBB, and transfer the mixture to the washed beads. If mineral oil was used in PCR-1, transfer the resuspended DNA (from **step 3, Subheading 3.4.2.**) directly to the beads without adding 5X WBB.
7. Incubate at 37°C (not in the magnetic rack) for 30 min with occasional agitation to resuspend the beads.

8. Place the tubes in the rack to sediment the beads, remove the supernatant, and wash three times with 200 μ L of **freshly prepared** 0.4 M NaOH, and then once with 200 μ L of TE.
9. Resuspend the beads in 40 μ L of H₂O, and transfer to PCR tubes.

3.4.4. PCR-2

1. The second-round, exponential PCR is carried out in a volume of 100 μ L containing the DNA template still attached to the beads. The reaction is the same whether damage is to be measured on the transcribed strand or the nontranscribed strand, since this specificity was determined by PCR-1. The components of the PCR are as for PCR-1 (**Subheading 3.4.2.**), except for: 50 pmol of each primer Oligo 3.2 and Oligo 5.2, 2 U of *Taq* polymerase, 2 μ Ci [α -³²P]-dATP.
2. Cycling conditions: An initial denaturation step of 2 min at 94°C and then 26 cycles of 94°C for 1 min, 60°C for 1 min (*see Note 14*), and 72°C for 1 min with a final incubation of 4 min at 72°C.
3. The PCR product is quantified, and the results expressed as described below.

3.4.5. Quantitation of ss-QPCR Product

One of two methods can be employed (*see Note 5*).

3.4.5.1. TCA PRECIPITATION

1. Transfer 40 μ L of PCR-2 product to 1.5-mL microcentrifuge tubes, and add 1 mL of 5% TCA mix.
2. Load Whatman GFC filters into a vacuum filtration manifold.
3. Rinse the filters with 1 mL of 5% (ice-cold) TCA mix.
4. Load the PCR-2 product/TCA mix onto the filters.
5. Wash the filters with 10 mL of 5% TCA mix (ice-cold) and 10 mL of absolute ethanol (ice-cold).
6. Air-dry the filters, place in scintillation vials, and add 5 mL of scintillation fluid.
7. Count on scintillation counter.

3.4.5.2. AUTORADIOGRAPHY AND DENSITOMETRIC SCANNING OR PHOSPHOR IMAGE ANALYSIS

1. Mix 10 μ L of PCR-2 product with 2 μ L of 6X agarose-gel loading buffer, and electrophorese in a 1.5% agarose gel (*see Note 15*).
2. Dry the gel on slab gel dryer, and expose to X-ray film or a phosphor image cassette.
3. Develop the film or cassette, and quantitate the bands by scanning or phosphor image analysis.

3.4.6. Expression of Results

1. The simplest way of expressing the results is as a percentage decrease of PCR product (compared to the untreated DNA control) as drug concentration increases (**Fig. 2**). Repair of damage is seen as a recovery of the amount of PCR product with time (*see Table 1*).

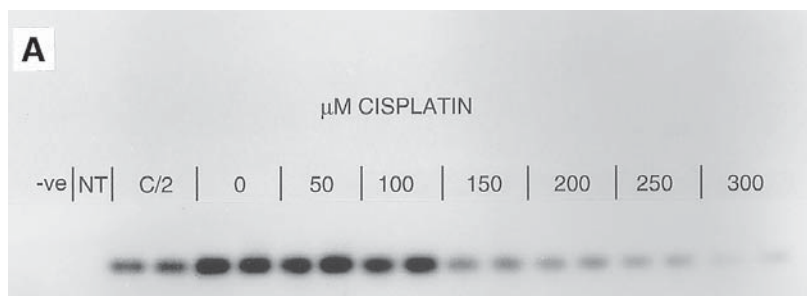


Fig. 2. (A) Autoradiograph showing 350 bp *N-ras* gene products of ss-QPCR on the transcribed strand (K562 cells treated with cisplatin for 16 h), after separation on a 1% agarose gel. (–ve) no genomic DNA, (NT) no *Taq* polymerase in the first-round PCR, (C/2) control sample containing half the test amount of (undamaged) DNA.

2. With relatively sequence-nonspecific DNA-damaging agents such as benzo-pyrene, UV, cisplatin, mechlorethamine, and so forth, the distribution of DNA adducts can be considered to be random, and the number of lesions per strand in the region defined by the primers can be calculated using the Poisson equation:

$$\text{Lesions/strand} = -\ln (Ad/A) \quad (1)$$

Where A is the amount of PCR product from the undamaged template and Ad is the amount from the damaged template. In this way, quantitative comparisons between drugs can be made.

3.4.7. Interpreting the Data

The results of measurement of DNA damage are shown in **Fig. 2**. Agarose-gel electrophoresis and autoradiography show that the product of the reaction is a single band of the expected size (**Fig. 2A**). In cisplatin-treated cells, the extent of the damage on the transcribed strand was consistently less than on the nontranscribed strand (**Fig. 2B**). The difference, 10–15%, was maintained over a wide range of drug concentrations and was similar in both cell lines.

In repair studies, initial damage was again greater on the nontranscribed strand, but was outside the 10–15% difference seen in damage studies (**Table 1**). This is probably owing to the kinetics of binding of cisplatin, since the repair studies included an exposure to drug of only 2 h rather than 16. The results show that both cell lines were capable of repairing damage, but there was no difference in the extent of repair of either strand in this gene segment, although the extent of repair was greater in the K562 cells.

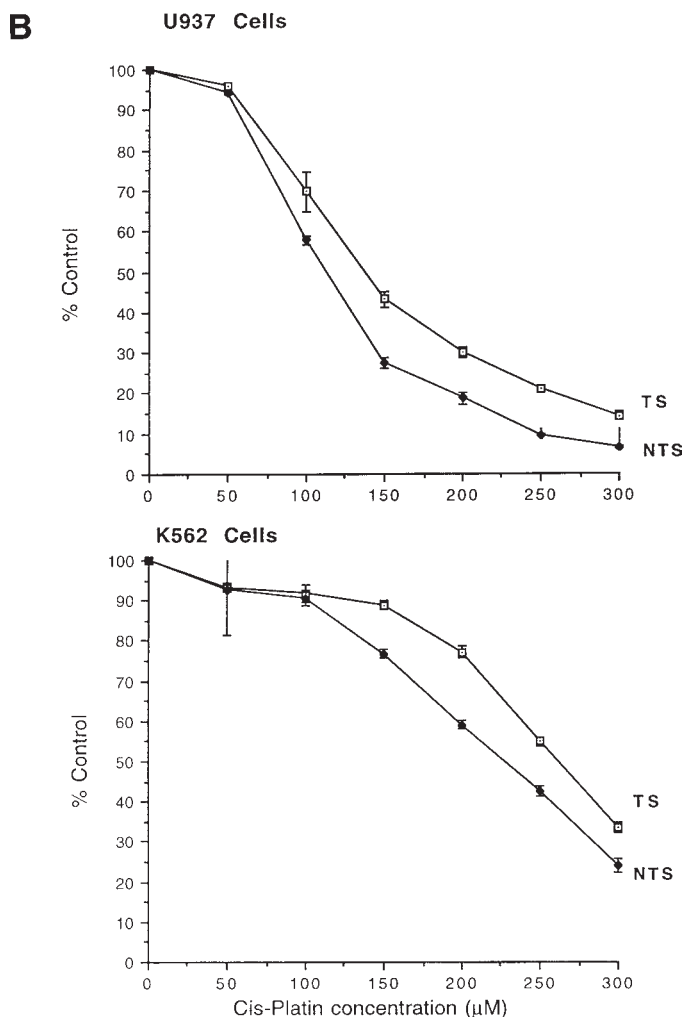


Fig. 2. **(B)** Percentage inhibition of ss-QPCR compared to untreated control cells, after 16 h treatment of K562 and U937 cells with various concentrations of cisplatin. TS = transcribed strand; NTS = nontranscribed strand.

4. Notes

1. The methods described are applicable to the study of DNA damage in any type of cell, not just transformed lines in culture. For example, damage can be studied in freshly isolated lymphocytes or cell preparations from solid tissue.
2. It has happened that primers that should have been biotinylated were not. Also we have had one batch of paramagnetic beads that did not bind efficiently. These are rare occurrences, but nevertheless possible and quite easy to test for:

Table 1
K562 and U937 Cells^a

	K562, <i>n</i> = 3		U937, <i>n</i> = 3	
	NTS	TS	NTS	TS
0 h repair (L/S) ^b	0.87 (±0.020)	0.80 (±0.018)	0.78 (±0.032)	0.57 (±0.020)
24 h repair (L/S)	0.13 (±0.004)	0.13 (±0.007)	0.34 (±0.007)	0.24 (±0.001)
% Lesions repaired	85.0	83.8	56.4	57.8

^aK562 and U937 cells were treated for 2 h with cisplatin (350 and 300 μ M, respectively) and allowed to repair for 24 h. (L/S, lesions/strand; NTS, nontranscribed strand; TS, transcribed strand).

^bLesions per strand were calculated according to the Poisson equation: $L/S = -\ln(Ad/A)$ where *A* is the amount of PCR product from undamaged template and *Ad* that from damaged template).

- a. Incubate 10 pmol of biotinylated primer with 5 μ L of washed beads in PCR buffer for 10 min, sediment the beads, and use the supernatant in a conventional PCR. Most of the biotinylated primers should be removed by the beads, and therefore, the quantity of product should be significantly reduced (usually by at least 50%) compared to the product obtained with uncaptured primers. It may not be completely reduced unless pure primers are used because the "failure sequence" oligonucleotides present (which would not bear the 5'-biotin), although being shorter can still participate in the PCR. It is also a good idea to perform this test in parallel using an unbiotinylated primer pair (of the same sequence) to control for any dilution or loss of primer that may occur when incubating with the beads; or
- b. Carry out a conventional PCR with 10 pmol of each primer, one of which being biotinylated. Then capture the product on the beads, and run the unbound fraction on an agarose gel. A reduction in product after capture (of around 50%) will be observed if all is functioning correctly. Again it is a good idea to run a parallel test with unbiotinylated primers.
3. 10X PCR buffer is supplied with the *Taq* polymerase. Of those we have tried, we have obtained good results with the Advanced Biotechnologies (UK) enzyme using their buffer IV and with Promega. Buffers that contain a small amount of detergent tend to give the best results.
4. The heated lid feature is very useful, since it removes the need to extract PCR samples with chloroform to remove the mineral oil.
5. Two methods are given for quantitation. TCA precipitation is the simplest and quickest. However, it is important to ensure beforehand (by electrophoresis and autoradiography) that the conditions of the PCR yield a single specific product of the expected size. If this is not the case, then electrophoresis and quantitation by densitometry or phosphor imaging would be appropriate.
6. Cells are treated in tissue-culture medium in the presence or absence of fetal calf serum according to the agent used and the length of incubation. Short incubations (e.g., 1–5 h) may be carried out in serum-free medium if the damaging agent is

also likely to react with serum proteins. Depriving the cells of serum will, however, disrupt their homeostatic environment, and this should be taken into account when designing experiments and interpreting results. We have found that the DNA-damaging effect of cisplatin is not significantly diminished when cells are treated in the presence of 5% serum, despite the known reactivity of cisplatin with protein. Different agents will no doubt behave in different ways, and whether or not to include serum will have to be determined empirically.

7. It might be found that during the treatment of adherent cultures some cells have detached themselves from the wells. If so, these should be harvested by centrifugation and washed as for suspension cells before adding them to the cells harvested from the wells by trypsinization.
8. When DNA is prepared from adherent cultures, the amount of the aqueous DNA-containing layer to be removed will depend on cell size, since a monolayer culture of large cells will obviously yield less DNA than a culture of smaller cells. With care, it is possible to remove up to 400 μ L without disturbing the interface.
9. The activity of the thermostable polymerase, efficiency of primers, quality of the genomic DNA template, and appropriate annealing temperatures can be determined by conventional PCR. If the primers are inefficient, the presence of formamide (1–10%) and/or DMSO (1–10%) in the PCR can often improve both efficiency and specificity.
10. The titration experiments are carried out under saturating PCR conditions. The ethidium-stained gels and autoradiographs may show some secondary bands in addition to the expected product. If their intensity is low, it is often found that they are not present at the lower cycle number used in the quantitative PCR experiments. This should be confirmed, since the generation of a single specific product is particularly important if quantitation by TCA precipitation is used (**Subheading 3.4.5.1.**).
11. The number of cycles to use to ensure that the reaction is stopped during the exponential phase must be determined empirically. The example here is appropriate for the *N-ras* region amplified.
12. “Hot start” is a simple procedure that can improve specificity. The idea is that all the components of PCR come together at a high temperature to avoid nonspecific priming at lower temperatures. The technique is as follows: The PCR mixture is prepared with all the components except *Taq* polymerase, and the tubes are placed in the cycler block. The machine is programmed so that before cycling starts, there will be an initial denaturation step of 3 min at 94°C followed by a 5-min pause at 80°C at which point the *Taq* polymerase is added. It is convenient to add the quantity of *Taq* required in a volume of 5 μ L. Place the tip of the pipet on the inside wall of the tube, lower it carefully to the bottom, expel the contents, and then remove the tip carefully keeping it pressed up against the tube wall. The cycling then commences with the first step being 1 min at 94°C.
13. The amount of $MgCl_2$ to use depends on the primers and the gene region being amplified. The concentrations used here are optimum for the *N-ras* primers used for ss-QPCR.

14. The annealing temperature will depend on the primers being used.
15. The percentage agarose to use in electrophoresis of PCR products depends on the size of the amplified DNA fragment—in this case 1.5% is appropriate for a 350-bp molecule.

Appendix

Human N-*ras*, intron 1 sequence: nontranscribed strand

```

CCTAAATCTG TCCAAAGCAG AGGCAGTGGG GCTTGAGGTA AGTTTATCTC
ATGCAGAGTG TTCGGCTTTG GGATGTGGAA TGTTTCAGGCG TTTCACTGAT
GCCAGAAATG GAGCAGAAATC TATCAGCTGG AGACAAAGGC CTTGGGCGGG
GGTCCTTCCA TTTGGTGCCT ACGTGGGGAG ATCTTGGAGA CAGAAGGGAG
AATGGGAAGG AGTTGCGGCC TGGAGGTTCC TGCTAGAGCT GAGAAGCCTT
CGGGGAGTAA TAGGAAGGGG GATCTCCATT GCTTAGGCTG AGGGCGGGGC
CCAAGGACTG TTGAAAAATA GCTAAGGATG GGGGTTGCTA GAAAACTACT
CCAGAAGTGT GAGGCCGATA TTAATCCGGT GTTTTTGCGT TCTCTAGTCA
CTTTAAGAAC CAAATGGGAAG GTCACACTAG GGTTTTCATT TCCATTGATT
ATAGAAGCTT TAAAGTACTG TAGATGTGGC TCGCAATTAA CCCTGATTAC
TGGTTTCCAA CAGGTTCTTG CTG

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Underlined bases: Sequence of primer 5.1tB (top) and sequence complementary to primer 3.1nB (bottom). Bases in bold type: Sequence of Oligo 5.2 (top) and sequence complementary to Oligo 3.2 (bottom).

References

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PCR-Based Assays for Strand-Specific Measurement of DNA Damage and Repair II

Single-Strand Ligation-PCR

Keith A. Grimaldi, Simon R. McAdam, and John A. Hartley

1. Introduction

The previous chapter describes strand-specific quantitative QPCR (ss-QPCR), which enables DNA damage and repair in short gene regions (~300 and upward) to be measured in a strand-specific manner. This chapter describes single-strand ligation PCR (sslig-PCR), which extends the previous method and allows damage and repair to be studied at the single-nucleotide level in single-copy genes in mammalian cells. The importance of this type of measurement is shown by the demonstration of a cell-specific adduct formed by the anticancer drug cisplatin, which is not formed when extracted DNA is treated *in vitro* (*1*).

1.1. Overview of the Protocol

The method of sslug-PCR is outlined in **Fig. 1**. As with ss-QPCR, it involves a first-round PCR using a single 5'-biotinylated primer, which defines the area of the gene to be investigated. Thirty cycles of linear amplification by PCR generate a family of single-stranded molecules of varying lengths for which the 5'-end is defined by the primer and the 3'-ends are defined by the positions of the DNA-drug adducts. In order to amplify exponentially these molecules, which are captured and isolated by binding to streptavidin-coated magnetic beads, a single-stranded, 5'-phosphorylated oligonucleotide is ligated to their 3'-OH ends using T4 RNA ligase. This oligonucleotide also bears a 3'-terminal amine group to block self-ligation. With both ends of the DNA molecules

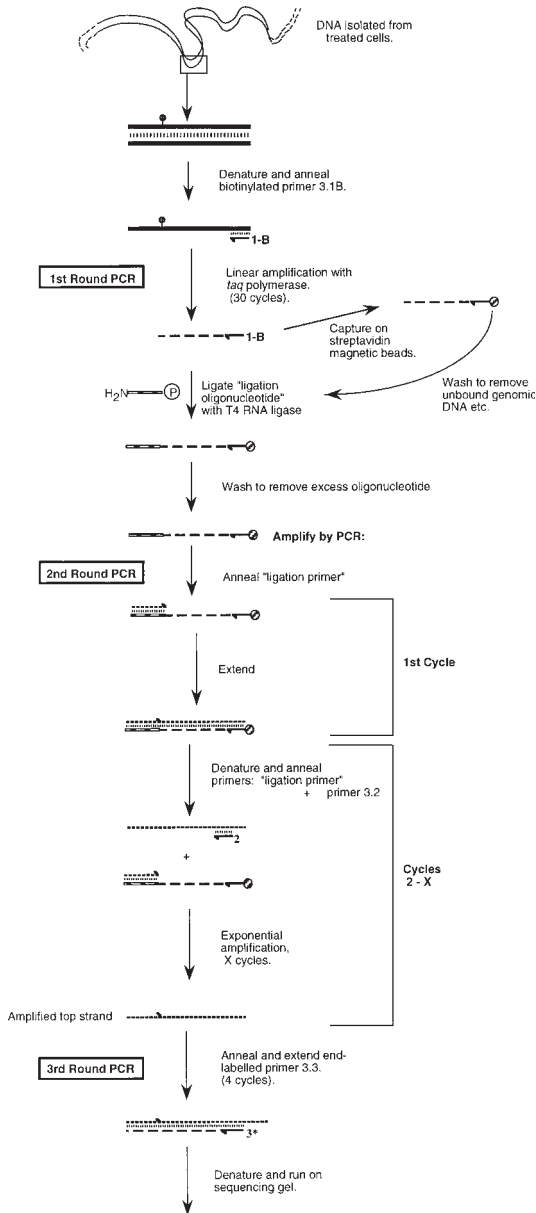


Fig. 1. Sslig-PCR method (as set up to measure lesions on the nontranscribed strand).

defined, they can then be exponentially amplified and detected. The sequence positions of the adducts are determined by electrophoresing the sslig-PCR products on a sequencing gel.

2. Materials

2.1. Cell and DNA Treatment

1. Cells in suspension or monolayer culture (*see Note 1*).
2. DNA damaging agent; e.g., UV, benzopyrene, cisplatin, and so forth.
3. 10X Teoa (for treatment of naked DNA): 250 mM triethanolamine, pH 7.2, 10 mM EDTA. Store at 4°C.
4. Drug stop solution: 0.6 M sodium acetate, pH 5.2.
5. Tissue-culture plates (6-well, 24-well, and/or Petri dishes).

2.2. DNA Isolation

1. Cell lysis buffer: 400 mM Tris-HCl, pH 8.0, 60 mM EDTA, 150 mM NaCl, 1% (w/v) sodium dodecylsulfate (SDS). Store at room temperature.
2. 5 M Sodium perchlorate (store at room temperature).
3. Chloroform.
4. 37 and 65°C water baths.
5. Rotary mixer.
6. Microcentrifuge.
7. Vacuum dryer.

2.3. Oligonucleotides

Oligonucleotides were obtained from Genosys (Pampisford, UK) or MWG Biotech, (Ebersberg, Germany). Store all oligonucleotides at -20°C. The oligonucleotide sequences will obviously depend on the region of the gene to be studied. Those described here can be used to study damage and repair in the 5'-region of the human *N-ras* gene and in a region of the 3'-end of the DNA Topoisomerase II (*TopoII*) gene (*see Appendix* for sequences).

1. To measure damage on the nontranscribed strand of *N-ras*: (intron 1 region): ras-int3.1B (5'-biotinylated): 5'-CAG CAA GAA CCT GTT GGA AAC CAG (*see Note 2*).
ras-int3.2: 5'-CCA GTA ATC AGG GTT AAT TGC GAG C.
ras-int3.3: 5'-GCG AGC CAC ATC TAC AGT AC.
2. To measure damage in the promoter region of the nontranscribed strand of *N-ras*: ras-prom3.1B (5'-biotinylated): 5'-GGA CAG ATT TAG GAC CAC AG (*see Note 2*).
ras-prom3.2: 5'-GAC CGG GAA AAA TGT TGG AGA.
N.B.: ras-prom3.2 is also used in the 3rd round PCR after labeling.
3. To measure damage on the transcribed strand of *TopoII*:
topo5.1B (5'-biotinylated): 5' CCT CCT GCT ACA CAT TTC CCA GAT (*see Note 2*).
topo5.2: 5' CCC AGA TGA AAC TGA AAT TAC AA.
topo5.3: 5' CAA ACC CAG TTC CTA AAA AG.
4. "Ligation Oligonucleotide"
5'-*p*-ATC GTA GAT CAT GCA TAG TCA TA-*n*: This oligonucleotide should

be supplied gel- or HPLC-purified. It must also be 5'-phosphorylated (*p*) and bear a 3'-terminal amine group (*n*) to block self-ligation (these modifications should be incorporated at synthesis).

5. "Ligation Primer"

5'-TAT GAC TAT GCA TGA TCT ACG AT: This oligonucleotide, which is complementary to the "ligation oligonucleotide", must be gel- or HPLC-purified.

2.4. 5'-End-Labeling of Oligonucleotides

Oligonucleotides are end-labeled with T4 polynucleotide kinase using Gibco BRL kits (Life Technologies, Paisley, UK) with forward reaction buffer.

1. [γ - ^{32}P]-ATP 10 $\mu\text{Ci}/\mu\text{L}$ (Amersham, Aylesbury, UK).
2. Forward buffer: 300 mM Tris-HCl, pH 7.8, 75 mM 2-mercaptoethanol, 50 mM MgCl_2 , 1.65 μM ATP.

2.5. PCR

1. *Taq* Polymerase (e.g., Perkin-Elmer, Beaconsfield, UK; Promega, Southampton, UK; Advanced Biotechnologies, Leatherhead, UK).
2. 10X PCR buffer (*see Note 3*): 200 mM $(\text{NH}_4)_2\text{SO}_4$, 750 mM Tris-HCl, pH 9.0, 0.1% (w/v) Tween.
3. 25 mM MgCl_2 (store at 4°C).
4. 10X dNTPs (Pharmacia, St. Albans, UK): make a mixture containing 2 mM concentration each of dATP, dGTP, dCTP, and TTP; store at -20°C.
5. Thermal cycler (e.g., MJ PTC-100 with heated lid; *see Note 4*).
6. Mineral oil (if thermal cycler is without heated lid facility).
7. PCR tubes—0.5 or 0.2 mL.

2.6. Capture and Ligation

1. Streptavidin M-280 Dynabeads (Dynal, Bromborough, UK) (*see Note 2*).
2. Magnet to capture beads. It should be able to accommodate at least six Eppendorf tubes (e.g., MPC-E6; Dynal UK).
3. 5X Washing and binding buffer (WBB): 25 mM Tris-HCl, pH 7.6, 5 mM EDTA, 5 M NaCl. Store at 4°C.
4. TE (pH 7.6): 10 mM Tris-HCl, pH 7.6, 1 mM EDTA.
5. PEG: 50% (w/v) PEG 8000. Store at 4°C.
6. 10X Ligation buffer: 0.5 M Tris-HCl, pH 8.0, 100 mM MgCl_2 , 10 mM hexamine (III) cobalt chloride, 100 $\mu\text{g}/\text{mL}$ bovine serum albumin (BSA), 200 μM ATP. Store at -70°C.

To make 1 mL:

500 μL	1 M Tris-HCl, pH 8.0
100 μL	1 M MgCl_2
10 μL	10 mg/mL BSA
2.68 mg	hexamine (III) cobalt chloride
2 μL	100 mM ATP
388 μL	H_2O .

7. T4 RNA Ligase (New England Biolabs, Hitchin, UK; activity = 20 U/ μ L; store at -20°C). (See Note 5.)

2.7. Sequencing Gel Electrophoresis

1. Sequencing gel-loading buffer: 96% (v/v) formamide (deionized), 20 mM EDTA, 0.03% (w/v) xylene cyanol, 0.03% (w/v) bromophenol blue.
2. Sequencing gel: 6% sequencing gels are prepared with Sequagel (National Diagnostics). Composition: 5.7% acrylamide, 0.3% *bis*-acrylamide, 8.3 M urea, 0.1 M Tris-borate, pH 8.3, 2 mM EDTA.
3. TEMED.
4. 50% (w/v) Ammonium persulfate (store at 4°C).
5. Sequencing gel apparatus: at least 60 cm in length, 20 cm in width, and 0.4 mm thick. (Suppliers include Kodak-IBI, Cambridge, UK, and Life Technologies).
6. 10X TBE: 0.9 M Tris-borate, 0.02 M EDTA (per L: 108 g of Tris base, 55 g of boric acid, 7.44 g of Na-EDTA).
7. Whatman DE81 and 3MM paper.
8. Gel dryer (suitable for acrylamide gels, e.g., Hoefer, San Francisco, CA).
9. Autoradiography cassettes ($43 \times 35 \text{ cm}^2$).
10. Autoradiography film.
11. Standard equipment for X-ray film development.

3. Methods

3.1. Treatment of Isolated DNA

1. Use 3 μg of DNA for each *sslig*-PCR.
2. Incubate the DNA with the drug for 1 h at 37°C in Teoa in a total volume of 50 μL in 1.5-mL microcentrifuge tubes.
3. Add 50 μL of 0.6 M sodium acetate “drug stop” solution, and precipitate the DNA with 3 vol of 95% ethanol.
4. Wash the DNA pellet twice with 1 mL of 75% ethanol (room temperature) and dry under vacuum.
5. Resuspend the DNA in 10 μL of deionized H_2O ready for PCR.

3.2. Treatment of Cells

3.2.1. Suspension Cultures

1. Count the cells, and resuspend at a density of 2×10^6 cells/mL in tissue-culture medium with or without fetal calf serum as required (see Note 6).
2. Add the required amount of drug (dissolved in tissue-culture medium or isotonic solution) to the wells of 24-well tissue-culture plates.
3. Add tissue-culture medium to make the volume up to 0.5 mL.
4. Add 0.5 mL of cell suspension (1×10^6 cells), and incubate at 37°C for the appropriate time.
5. Transfer the cells to 1.5-mL microcentrifuge tubes, rinse the wells with 0.4 mL of tissue-culture medium, and add the rinse to the tubes. Spin for 5 min at 270g, 4°C .

6. Remove the supernatant and wash the cells—by resuspending and spinning—with 1 mL of tissue-culture medium. Repeat twice more.
7. After the washings remove the supernatant. At this point, the cell pellet may be stored at -20°C until DNA isolation. For repair experiments, resuspend the cells in 1 mL of tissue-culture medium supplemented with fetal calf serum, transfer to a fresh 24-well plate, and incubate at 37°C for the appropriate times before harvesting the cells.

3.2.2. Adherent Cells

1. Grow cells to almost confluence in 2-cm diameter wells.
2. Treat with the drug as for suspension cells, except first mix the drug in 1 mL of tissue-culture medium to avoid adding concentrated drug directly to the cells.
3. Incubate as for suspension cells.
4. Remove the drug medium, and gently wash the cells three times with 1 mL of fresh tissue culture medium. (See Note 7.)
5. If repair experiments are to be carried out, add tissue-culture medium with serum, and incubate for the appropriate times.
6. Harvest the cells by trypsinization, and spin as for suspension cells. These cells may be stored at -20°C .

3.3. DNA Isolation

1. Resuspend the cell pellet in 500 μL of cell lysis buffer.
2. Add 120 μL of 5 *M* sodium perchlorate.
3. Incubate at 37°C for 20 min, mixing occasionally.
4. Transfer to a 65°C water bath, and incubate for 20 min with occasional mixing by inversion.
5. Add 700 μL of chloroform precooled to -20°C .
6. Mix by rotation for 20 min at room temperature.
7. Spin in microcentrifuge at 11,600g for 10 min.
8. Remove half (310 μL ; equivalent to 5×10^5 cell from suspension cultures) the upper aqueous layer, transfer to a fresh 1.5-mL microcentrifuge tube, and add 620 μL of absolute ethanol (kept at -20°C) to precipitate the DNA. (See Note 8).
9. Spin at top speed in a microcentrifuge for 20 min, and wash the DNA pellet twice with 1 mL of 75% ethanol (kept at room temperature).
10. Dry the DNA pellet under vacuum.
11. Resuspend the pellet in 50 μL of H_2O (see Notes 9 and 10). Use 10 μL /PCR.

3.4. *sslig*-PCR (see Note 11)

3.4.1. PCR-1

1. The single primer used in the first round determines on which strand the damage will be measured (i.e., using a primer complementary to the transcribed strand will detect damage on the transcribed strand). First-round “linear” PCR is carried out in a volume of 40 μL containing: 0.6 pmol of 5'-biotinylated primer (e.g., ras-

- int3.1B); 1 U of *Taq* polymerase (see **Note 12**); 200 μ M each dATP, dGTP, dCTP, TTP; 2.5 mM MgCl_2 (see **Note 13**); 4 μ L of 10X PCR buffer. If necessary, add 40 μ L of mineral oil overlay.
2. Place the tubes in the thermal cycler, and carry out the following program: an initial denaturation step of 3 min at 94°C and then 29 cycles of 94°C for 1 min, 60°C for 1 min (see **Note 14**), and 72°C for 1 min + a 1 s extension/cycle. This is followed by a final incubation of 4 min at 72°C.
 3. If mineral oil was used, this needs to be removed as follows: Add 60 μ L of H_2O and 100 μ L of water-saturated chloroform. Spin the tubes briefly, remove the upper aqueous layer, and transfer to 1.5-mL microcentrifuge tubes. Add a further 100 μ L of H_2O to the original PCR tubes, spin again, remove the aqueous layer, and add to the 1.5-mL microcentrifuge tubes. Precipitate the DNA with ethanol and, after drying, resuspend in 50 μ L of 1X WBB.

3.4.2. Capture of PCR Products

1. Transfer streptavidin-coated Dynabeads to a 1.5-mL microcentrifuge tube. Use 5 μ L per PCR plus an extra 5 μ L (e.g., for 10 reactions, transfer 55 μ L of beads).
2. Place the tube in the magnetic rack to sediment the beads (for ~30 s) and then remove the supernatant—keep the tube in the magnetic rack!
3. Remove the tube from the magnetic rack, and resuspend the beads in 200 μ L of 1X WBB. Return the tube to the rack to sediment the beads, and then remove the supernatant. Repeat this washing process once more.
4. Resuspend the beads in 1X WBB using 40 μ L/PCR (i.e., for 10 tubes resuspend in 400 μ L). Mix well, and transfer 40- μ L aliquots to 1.5-mL microcentrifuge tubes.
5. Place the tubes in the rack to capture the beads, and then remove the supernatant. The beads are now ready for the addition of the PCR mix.
6. To the 40 μ L PCR mix, add 10 μ L of 5X WBB, and transfer the mixture to the washed beads. If mineral oil was used in PCR-1, transfer the resuspended DNA (from **step 3, Subheading 3.4.1.**) directly to the beads without adding 5X WBB.
7. Incubate at 37°C (not in the magnetic rack) for 30 min with occasional agitation to resuspend the beads.
8. Place the tubes in the magnetic rack, remove the supernatant, and wash the beads three times with 200 μ L of TE.
9. Resuspend the beads in 50 μ L of H_2O , and spin briefly in a microcentrifuge to bring all the liquid to the bottom of the tubes.

3.4.3. Ligation of “Ligation Oligonucleotide”

1. Prepare the ligation mix. The ligation is carried out in a volume of 10 μ L. Prepare sufficient mix to give 10 μ L more than required (i.e., for 10 tubes prepare a mix of 110 μ L). The composition of the mix (per tube) is as follows:
 - 5 μ L of 50% PEG
 - 1 μ L of ligation oligonucleotide (20 pmol/ μ L)
 - 1 μ L of 10X ligation buffer

- 2 μL of H_2O
- 1 μL of T4 RNA Ligase (20 U/ μL)

2. Place the tubes containing the bead suspension in the magnetic rack to sediment the beads. Remove the supernatant.
3. Resuspend the beads in 10 μL of ligation mix, and ligate overnight at room temperature.
4. After ligation, add 180 μL of TE, and place the tubes in the magnetic rack.
5. Remove the supernatant, and wash the beads three times with 200 μL of TE.
6. Resuspend the beads in 40 μL of H_2O ready for PCR-2.

3.4.4. PCR-2 and PCR-3

1. The second-round PCR is carried out in a volume of 100 μL containing the DNA template on the beads. Two primers are used: “ligation primer” and gene-specific primer (e.g., ras-int3.2). In the N-ras (intron 1) example, to measure adducts on the nontranscribed strand, ras-int3.1B is used in PCR-1, and therefore, ras-int3.2 will be used in PCR-2 (and ras-int3.3 in PCR-3).
2. Transfer the beads, suspended in 40 μL H_2O , to PCR tubes containing the PCR mix. The reaction composition is as follows: 10 pmol of ras-int3.2; 10 pmol of “ligation primer”; 2.5 U of *Taq* polymerase; 200 μM each dATP, dGTP, dCTP, TTP; 2.5 mM MgCl_2 (see **Note 13**); 10 μL of 10X PCR buffer.
3. The cycling conditions are: an initial denaturation at 94°C for 5 min, then *X* cycles of 94°C for 1 min, 58°C for 1 min (see **Note 14**), 72°C for 1 min, + a 1-s extension/cycle with a final 5-min step at 72°C. The number of cycles (*X*) in this step has to be determined empirically for each set of primers. It generally falls between 22 and 28 cycles (see **Note 15**).
4. PCR-3 is carried out immediately after PCR-2 is finished. Add to the tubes 10 μL of PCR mix containing: 1 μL of 10X PCR buffer, 5 μL (5 pmol) of ^{32}P 5'-end-labeled primer ras-int3.3 (see below, **Subheading 3.4.6.**), 1 U of *Taq* polymerase, 1 μL of 10X dNTP mix, 2.5 mM MgCl_2 (see **Note 13**).
5. Perform a further 4 cycles: 94°C for 1 min, 64°C for 1 min (see **Note 14**), and 72°C for 1 min with a final 5 min step at 72°C.
6. Spin the PCR tubes briefly in a microcentrifuge (remove mineral oil at this point if necessary) and transfer the supernatant to 1.5 mL microcentrifuge tubes. Rinse the PCR tubes with 100 μL of H_2O , spin, and add to the microcentrifuge tubes. Precipitate the DNA with 3 vol of 95% ethanol (kept at –20°C) and dry under vacuum.

3.4.5. Sequencing Gel

1. Resuspend the DNA (now radioactive) in 5 μL of sequencing gel loading buffer, and electrophorese in a 6% acrylamide sequencing gel at 2500–3000 V (see **Note 16**).
2. Dry the gel onto Whatman 3MM paper, supported by a layer of Whatman DE 81 paper to bind the shorter fragments, which will pass through the 3MM.
3. Expose the gel to X-ray film. An overnight exposure is usually sufficient to give a signal. Sometimes an intensifying screen may be required, and if so, the film is exposed at –70°C.

3.4.6. Oligonucleotide 5'-End-Labeling

1. Five pmol of end-labeled primer (e.g., *ras-int3.3*) are required for each tube in PCR-3. For 10 samples, label 55 pmol: In a 1.5-mL microcentrifuge tube add 12.5 μL of H_2O , 5.5 μL of *ras-int3.3* (at a concentration of 10 pmol/ μL), 1 μL of T4 polynucleotide kinase (5 U/ μL), 1 μL of [$\gamma\text{-}^{32}\text{P}$]-ATP (10 $\mu\text{Ci}/\mu\text{L}$), 5 μL of 5X reaction buffer.
2. Incubate at 37°C for 30 min, add 25 μL of H_2O , and separate unincorporated nucleotide from the labeled oligonucleotide either by ethanol precipitation or by using a spin column (e.g., Bio-Spin-6; Bio-Rad). Use the labeled oligonucleotide as directed in **Subheading 3.4.4**.

3.4.7. Interpreting the Data (see **Notes 17 and 18**)

1. The resulting autoradiograph will be similar to that of a sequencing gel, except that the bands will not be regularly spaced. **Figures 2 and 3** show the results from treating cells with cisplatin and tallimustine (a novel sequence-selective cytotoxic agent). The bands on the autoradiograph correspond to the position of the adducts in the sequence of the intron 1 region of the *N-ras* gene (**Fig. 2**), the promoter region of the *N-ras* gene and the 3'-end of the human *TopoII* gene (**Fig. 3**; see **Appendix** for sequences). **Figure 2** shows that cisplatin forms adducts mainly at runs of two or more guanines and that a cell-specific adduct is formed at the sequence 5'-TACT. Tallimustine on the other hand is highly sequence-specific, and binds preferentially to the sequence 5'-TTTTGA as shown in **Fig. 3**. In the *N-ras* gene, adducts are formed at only one of the two 5'-TTTTGA sites. The results shown in **Fig. 3** are from a repair experiment, and they highlight the importance of studying repair at the nucleotide level. DNA from the same cell treatment was used as template to look at lesions in the promoter region of the *N-ras* gene and in a region of the *TopoII* gene. In the *N-ras* gene, the tallimustine adduct is not repaired, and in fact, the adduct intensity increases during the repair incubation, whereas in the *TopoII* gene, ~50% of the adducts are repaired.
2. The intensity of the bands is dose-dependent, and the amount of damaging agent to use in the treatment is important; it should produce at most 1 adduct/strand in the region studied. The reason for this is that the *Taq* polymerase will be blocked by the first adduct it encounters, and therefore, if more than 1 adduct per strand is present, the final result will not give an accurate representation of the distribution of adducts.
3. There are several ways to determine the nucleotide position of the adduct. Generally, an overexposure of the autoradiograph will give enough background to produce a "ladder" of bases, so that the position of the adduct bands can be counted from the primer. This is the easiest way—the size of the "ligation oligonucleotide" must be taken into account, since this will add 23 bases (in the example described in this chapter) to the position of the adduct. An alternative method is to run a sequencing ladder of a stretch of DNA of known sequence, so that the distance of the adducts from the primer can be precisely determined. Of course the sequencing must be carried out using ^{32}P as in the *sslig-PCR* and not using ^{35}S .

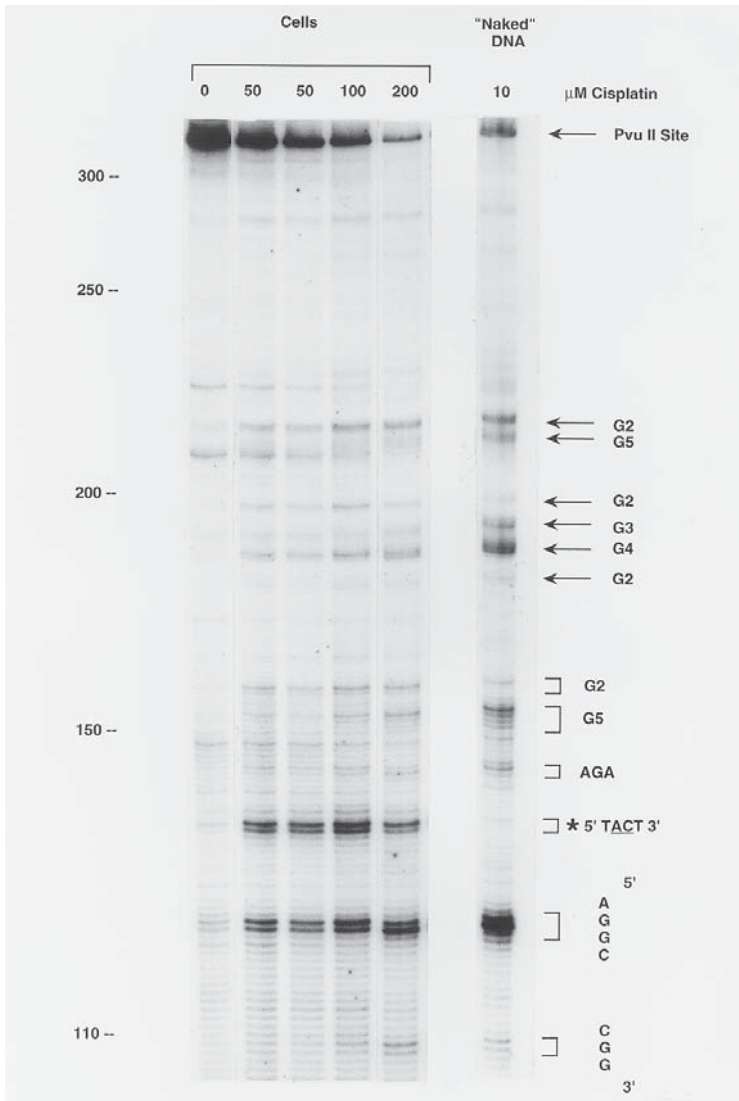


Fig. 2. Concentration-dependent formation of cisplatin adducts on the non-transcribed strand of the *N-ras* gene (intron-1). Cisplatin adduct formation is compared in cells and "naked" DNA. The bands on the autoradiograph represent adducts formed in cells treated for 18 h with cisplatin. DNA extracted from cells was cut with *Pvu*II before sslig-PCR to create a defined stop site on lesion-free templates (see **Note 9**). The asterisk next to the sequence 5'-TACT indicates cisplatin lesion sites, which were cell-specific and were not found in treated "naked" DNA.

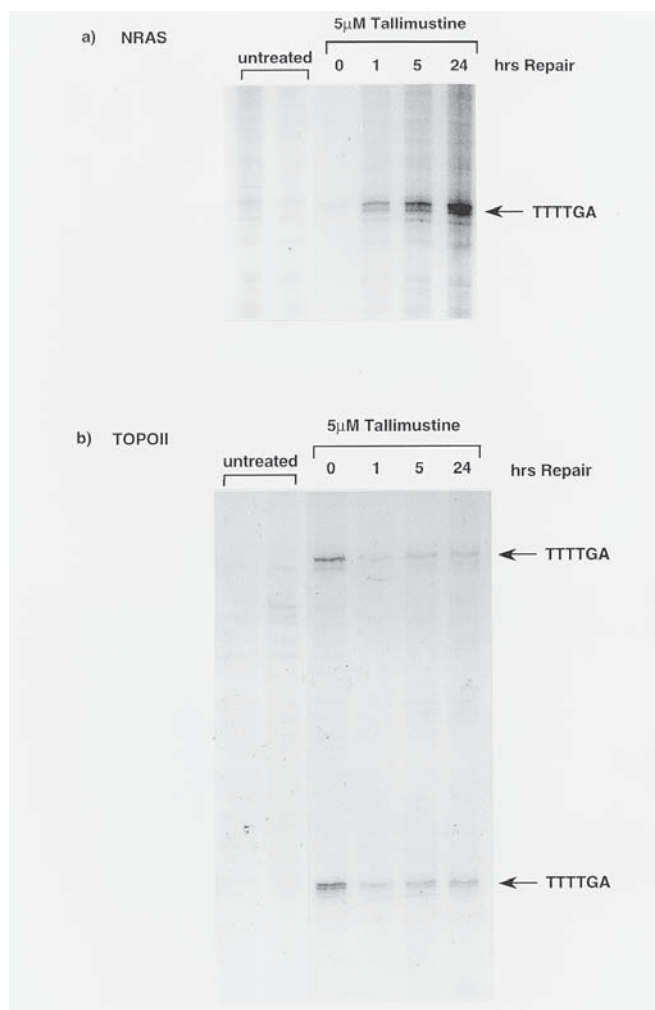


Fig. 3. Sslig PCR: Damage and repair of tallimustine adducts in the promoter region of the human *N-ras* gene (nontranscribed strand) and in the 3' region of the human *TopoII* gene (transcribed strand). Li-Fraumeni syndrome (LFS) fibroblasts were treated for 2 h with 5 μ M tallimustine, washed free of drug, and allowed to repair for 0, 1, 5 and 24 h. DNA was extracted and the adducts, which formed at 5'-TTTTGA sites, were detected with sslig-PCR. In the *N-ras* gene, an adduct was formed at only one of the two 5'-TTTTGA sites; there was no repair, and adduct intensity actually increased. In the *TopoII* gene, there was repair which was approx 50% complete after 1 h, and remained at this level throughout the entire 24-h repair period (similar repair was seen on the nontranscribed strand of the *TopoII* gene).

4. Sslig-PCR can also be used for genomic sequencing, so this can also be used as a method of assigning the position of the adduct, although this is a labor-intensive method. Genomic sequencing reactions are performed on isolated DNA and the sslig-PCR carried out together with the drug-treated samples. The sequencing samples are then run on the gel alongside the treated samples. The description of the genomic sequencing reactions is beyond the scope of this chapter, but details can be found in Pfeifer et al. (2).
5. Quantitation of the intensity of individual adducts can be carried out by image densitometry or phosphor imaging.

4. Notes

1. The methods described are applicable to the study of DNA damage in any type of cell, not just transformed lines in culture. For example, damage can be studied in freshly isolated lymphocytes or cell preparations from solid tissue.
2. It has happened that primers that should have been biotinylated were not. Also we have had one batch of paramagnetic beads that did not bind efficiently. These are rare occurrences, but nevertheless possible and quite easy to test for:
 - a. Incubate 10 pmol of biotinylated primer with 5 μ L of washed beads, in PCR buffer for 10 min. Sediment the beads, and use the supernatant in a conventional PCR. Most of the biotinylated primers should be removed by the beads, and therefore, the quantity of product should be significantly reduced (usually by at least 50%) compared to the product obtained with uncaptured primers. It may not be completely reduced unless pure primers are used because the “failure sequence” oligonucleotides present (which would not bear the 5'-biotin), although being shorter can still participate in the PCR. It is also a good idea to perform this test in parallel using an unbiotinylated primer pair (of the same sequence) to control for any dilution or loss of primer that may occur when incubating with the beads; or
 - b. Carry out a conventional PCR with 10 pmol of each primer, one of which being biotinylated. Then capture the product on the beads, and run the unbound fraction on an agarose gel. A reduction in product after capture (of around 50%) will be observed if all is functioning correctly. Again it is a good idea to run a parallel test with unbiotinylated primers.
3. 10X PCR buffer is supplied with the *Taq* polymerase. Of those we have tried, we have obtained good results with the Advanced Biotechnologies (UK) enzyme using their buffer IV and Promega. Buffers that contain a small amount of detergent tend to give the best results.
4. The heated lid feature is very useful as it removes the need to extract PCR samples with chloroform to remove the mineral oil.
5. We have had one batch of T4 RNA ligase apparently contaminated with exonuclease activity, which removes the 3'-terminal amine block on the “ligation oligonucleotide.” The result on the autoradiograph is a ladder of intense bands at intervals of 20–23 bp owing to serial self-ligation (the “ligation oligonucleotide” is 23 bp). The efficiency of the ligation step can be tested using donor and accep-

tor nucleotides as described in Tessier et al. (3). If exonuclease is present, the labeled oligonucleotide will be degraded.

6. Cells are treated in tissue-culture medium in the presence or absence of fetal calf serum according to the agent used and the length of incubation. Short incubations (e.g., 1–5 h) may be carried out in serum-free medium if the damaging agent is also likely to react with serum proteins. Depriving the cells of serum will however disrupt their homeostatic environment, and this should be taken into account when designing experiments and interpreting results. We have found that the DNA-damaging effect of cisplatin is not significantly diminished when cells are treated in the presence of 5% serum despite the known reactivity of cisplatin with protein. Different agents will no doubt behave in different ways, and whether or not to include serum will have to be determined empirically.
7. It might be found that during the treatment of adherent cultures some cells have detached themselves from the wells. If so these should be harvested by centrifugation and washed as for suspension cells before adding them to the cells harvested from the wells by trypsinization.
8. When DNA is prepared from adherent cultures, the amount of the aqueous DNA-containing layer to be removed will depend on cell size, since a monolayer culture of large cells will obviously yield less DNA than a culture of smaller cells. With care it is possible to remove up to 400 μ L without disturbing the interface.
9. It is often useful to digest the DNA after isolation and before *sslig-PCR* with a restriction enzyme that cuts around 200–400 bases upstream of the primers used. This creates a “full-length” stop site where *Taq* polymerase will be stopped in the absence of downstream damage. As shown in **Fig. 2**, the intensity of this band (the *PvuII* site) reduces as DNA-damaging agent concentration increases because of the downstream DNA adducts. This reduction can be quantitated by densitometry or phosphor imaging and the values can be used with the Poisson equation to give an estimate of the average number of lesions per strand (*see* Chapter 19, **Subheading 3.4.6.**).
10. The restriction-digested DNA is also useful in the initial stages of setting up the *sslig-PCR* for new gene regions. The intensity of the “full-length” band will give a measure of the efficiency of the *sslig-PCR* when using untreated DNA.
11. The activity of the thermostable polymerase, efficiency of primers, quality of the genomic DNA template, and appropriate annealing temperatures can be determined by conventional PCR. If the primers are inefficient, the presence of formamide (1–10%) and/or DMSO (1–10%) in the PCR can often improve both efficiency and specificity.
12. “Hot start” is a simple procedure that can improve specificity. The idea is that all the components of PCR come together at a high temperature to avoid nonspecific priming at lower temperatures. The technique is as follows: The PCR mixture is prepared with all the components except *Taq* polymerase and the tubes are placed in the cycler block. The machine is programmed so that before cycling starts there will be an initial denaturation step of 3 min at 94°C followed by a 5-min pause at 80°C at which point the *Taq* polymerase is added. It is convenient to add

the quantity of *Taq* required in a volume of 5 μ L. Place the tip of the pipet on the inside wall of the tube, lower it carefully to the bottom, expel the contents, and then remove the tip carefully keeping it pressed up against the tube wall. The cycling then commences with the first step being 1 min at 94°C.

13. The amount of $MgCl_2$ to use depends on the primers and the gene region being amplified. The concentration used here is optimum for the *N-ras* gene region studied.
14. The annealing temperature will depend on the primers being used.
15. The optimum number of cycles will give the best signal/noise ratio. This falls within the exponential range of the reaction which is important when quantitation of individual adducts is to be carried out—especially in repair experiments.
16. Gels should be as long as possible, preferably 80 cm rather than the usual 40-cm gels used for sequencing. This is because ^{32}P is used as the isotope that gives less resolution compared to ^{35}S as used in dideoxy sequencing. If long gel equipment is not available, the shorter gels can be run for longer, running the shorter fragments off the bottom of the gel. It should be remembered that the buffer in the lower chamber will become radioactive.
17. The main reason for poor resolution of bands on the autoradiograph is inadequate drying of the sequencing gel before autoradiography.
18. A common problem encountered when setting up ssIig-PCR is too much background, that is, nonspecific bands on the autoradiograph caused either by spontaneous premature termination of primer extension, or nonspecific primer binding, in the first-round PCR. There are several possible reasons why this may occur:
 Nonoptimal $MgCl_2$ concentration especially in the first round, linear PCR;
 Annealing temperature is too low;
 Too much genomic DNA template in the first round PCR;
 Too many cycles in the second-round PCR (as with all PCR assays, a small level of background is inevitable. This level will be exaggerated if too many cycles are used in the exponential second-round PCR).

Appendix

1. Human *N-ras*, intron 1 sequence: nontranscribed strand

5'

```

484 CCTAAATCTG TCCAAAGCAG AGGCAGTGGG GCTTGAGGTA AGTTTATCTC
434 ATGCAGAGTG TTCCGCTTTG GGATGTGGAA TGTTTCAGGCG TTTCAGTGAT
384 GCCAGAAATG GAGCAGAAATC TATCAGCTGG AGACAAAGGC CTTGGGCGGG
334 GGTCTTCCA TTTGGTGCCT ACGTGGGGAG ATCTTGAGAG CAGAAGGGAG
284 AATGGGAAGG AGTTGCGGCC TGGAGGTTCC TGCTAGAGCT GAGAAGCCTT
234 CGGGGAGTAA TAGGAAGGGG GATCTCCATT GCTTAGGCTG AGGGCGGGG
184 CCAAGGACTG TTGAAAAATA GCTAAGGATG GGGGTTGCTA GAAAACTACT
134 CCAGAAGTGT GAGGCCGATA TTAATCCGGT GTTTTTGCGT TCTCTAGTCA
84 CTTTAAGAAC CAAATGGAAG GTCACACTAG GGTTCATT TCCATTGATT
34 ATAGAAGCTT TAAAGTACTG TAGATGTGGC TCGCAATTAA CCCTGATTAC
TGGTTTCCAA CAGGTTCTTG CTG

```

The complementary sequence of primer *ras-int3.3* is underlined, numbering starts from the first base (C) and corresponds to that in **Fig. 2**.

2. Human *N-ras*, promoter region sequence: non-transcribed strand

5'

TTCTAGGACC CGGTTTCTTT TACTGATTTA AAAACAAAAC AAAAAAAAAAT
 AAAAAAGTTG TGCCTGAAAT GAATCTTGTT TTTT TTTTAT AAGTAGCCGC
 CTGGTTACTG TGTCTGTAA AATACAGACA TTGACCCCTG GTGTAGCTTC
 TGTTCAACTT TATATCACGG GAATGGATGG GTCTGATTTT TTGGCCCTCT
 TCTTGAATTG GCCATATACA GGGTCCCTGG CCAGTGGACT GAAGGCTTTG
 TCTAAGATGA CAAGGGTCAG CTCAGGGGAT GTGGGGGAGG GCGGTTTAT
 CTTCCCCCTT GTCGTTTGAG **GTTTGATCT** CTGGGTAAAG AGGCCGTTTA
 TCTTTGTAAC CACGAAACAT TTTTGCTTTC TCCAGTTTTC TGTTAATGGC
 GAAAGAATGG AAGCGAATAA AGTTTTACTG **ATTTTGAGA** CACTAGCACC
 TAGCGCTTTC ATTATTGAAA CGTCCCGTGT GGGAGGGGCG GGTCTGGGTG
 CGGCCTGCCG CATGACTCGT GGTTCCGAGG CCCACGTGGC CGGGGCGGGG
 ACTCAGGCGC CTGGGGCGCC GACTGATTAC GTAGCGGGCG GGGCCGAAG
 TGCCGCTCCT TGGTGGGGGC TGTTTCATGGC GGTTCGGGG TCTCCAACAT
TTTCCCGGT CTGTGGTCCT AAATCTGTCC AAAGCAGAGG CAGTGGAGCT

The complementary sequence of primer *ras-prom3.2* is underlined, and potential target sequences for tallimustine are in bold.

3. Human *TopoII*, genomic sequence (3'-end): transcribed strand

5'

GCGGCGATTC TTGGTTTTGG CAGGATCAGG CTTTGTAGAG ACACCAGAAT
 TCAAAGCTGG ATCCCTTTTA GTTCC**TTTTG** GGGCAGCCCT TTT**TTTTGGCA**
 CCGGTAGTGG AGGTGGAAGA CTGCAAGCAT CCTCTGTGTT AGCCTCCAAG
 TGCTGGATAC AGCATGAGCC ACTCTGCCTG ACTTGTGTTG **TTTTTGACTA**
 GTCAGTGCAG TAGTGAGAAG GAGGGAAAAG AGTACAACAA GGAGTTGATC
 TGTAACAGAA CAATCAATAG AGATACTTAC **TGTTTTGGAA** CAGCCTTGAC
 TACTAA**TTTT** **GATTATAAAG** ATCGAAAAGA AATTGCTTCC AATTGAAAAA
 CATTTAATCT GTAATATCAA TAGTAAG**TTT** **TGGCAATAAA** AAAATTGAAA
 TAGACACGTG GAAACCAGTT AGGTAATTGC AACATTAACC CATCTCAAAG
 ATTTAGGCTT ACTTTTTGCT GCTGTCTTCT TCACTGTCAC ATT**CTTTT**
GGAAGTGGT TTGTAATTTC AGTTTCATCT GGGAAATGTG TAGCAGGAGG

The complementary sequence of primer *topo5.3* is underlined, and potential target sequences for tallimustine are in bold.

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Gene-Specific and Mitochondrial Repair of Oxidative DNA Damage

R. Michael Anson and Vilhelm A. Bohr

1. Introduction

DNA repair is a heterogeneous process. The efficiency of repair differs not only with lesion type, but also with genomic location. Protein coding regions of the genome are repaired more efficiently than noncoding regions in a process termed preferential repair. Furthermore, for many lesions, there is a hierarchy even within the coding regions. A process termed transcription-coupled repair allows active genes to be repaired more rapidly than inactive genes, and the transcribed strand to be repaired with greater efficiency than the nontranscribed strand (*1*).

To measure differences in the rate of repair between different regions of the genome and different strands within a given region, the Southern blot gene-specific DNA damage and repair assay is frequently used (*1*). The principle behind the assay is to use a DNA repair enzyme as a tool to nick a restricted DNA fragment at the site of damage. In a denaturing agarose gel, the cleaved strand migrates further than the full-length restriction fragment (**Fig. 1**). This loss of full-length, damaged DNA from the main band, detected by Southern blot, allows quantitation of the undamaged DNA. Film (or PhosphorImager) exposures are chosen that give a linear relationship between band intensity and the DNA content of the band. A random distribution of damage in a given restriction fragment is assumed, which allows the use of Poisson equations in determination of the average number of adducts per strand. The assay has been successfully applied to many different lesions, including UV damage (the major product of which is the pyrimidine dimer), 4-nitroquinoline-*N*-oxide, *N*-acetaminofluorene, benzopyrene diol epoxide, cisplatin, various alkylating agents, and most recently, several types of oxidative DNA damage

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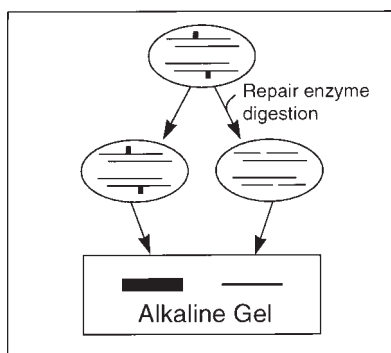


Fig. 1. The principle behind the Southern-blot gene specific damage and repair assay. A DNA repair enzyme is used as a tool to nick a restricted DNA fragment at the site of damage. In a denaturing agarose gel, the cleaved strand migrates further than the full-length restriction fragment. The band is detected by Southern blot.

(2,3). In general, the assay allows measurement of any type of damage that can be converted to a single-stranded nick either chemically or by use of a repair enzyme. For alkali-sensitive damage, aliquots of the sample may be run on neutral and alkaline gels, and the band intensities normalized to a damage-free internal standard. The intensity of the alkaline band relative to the native band may then be used to calculate the number of alkali-sensitive sites.

1.1. Oxidative DNA Damage in Nuclear and Mitochondrial DNA

Aerobic organisms are exposed to reactive oxygen species (ROS) as an unavoidable consequence of normal metabolism, and reaction of the organism's DNA with ROS causes many potentially mutagenic or lethal lesions, including strand breaks, abasic sites, and oxidized bases. The development of methods to measure the repair of oxidative DNA lesions, particularly at the gene-specific level, is the subject of much effort in the DNA repair field at this time. The difficulties that are encountered lie not only in the detection of the lesions, but also in their induction: methods commonly used to induce oxidative stress experimentally frequently cause damage to cell membranes or other components in excess of the damage to the DNA itself.

Within the cell, the mitochondrial transport chain is a major site of ROS production, and the close proximity of the mitochondrial DNA makes it a likely target for damage. The accumulation of such damage is thought to lead to an eventual loss of mitochondrial function, and to play a role in carcinogenesis and cellular aging. One of the most common oxidative lesions is 8-oxo-7-hydrodeoxyguanosine (8-oxo-dG), a potentially mutagenic lesion that will often adopt the syn conformation, allowing it to mis-pair with deoxyadenosine. Steady-

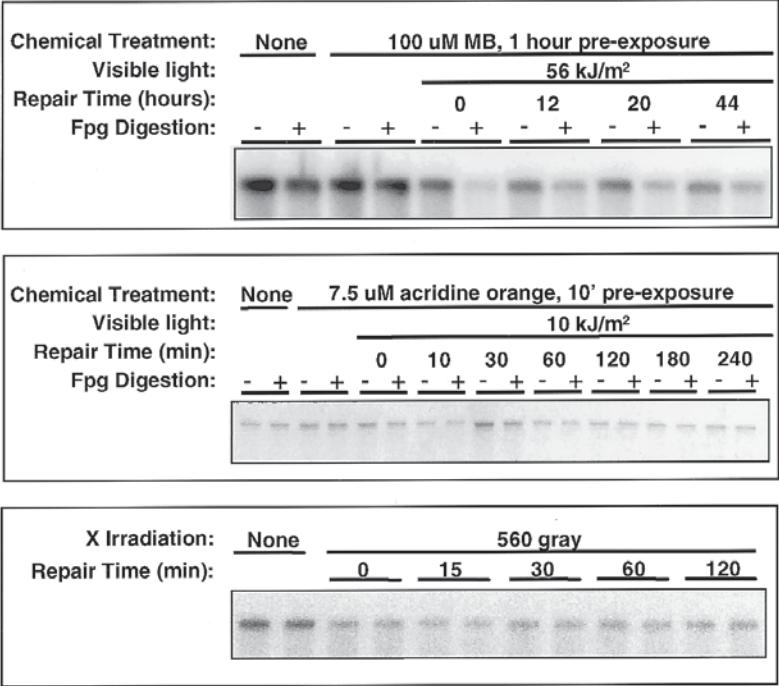


Fig. 2. Sample data obtained using the Southern-blot gene specific damage and repair assay. (Top), Damage and repair of Fpg-sensitive base damage in mitochondrial DNA following treatment of cells with photoactivated methylene blue. (Middle), Damage and repair of Fpg-sensitive base damage in mitochondrial DNA following treatment of cells with photoactivated acridine orange. (Bottom) Damage and repair (measured by comparison with DNA from untreated cells) of alkali labile sites and strand brakes following treatment of cells with X irradiation.

state levels of 8-oxo-dG are often reported to be 10-fold higher in mitochondrial DNA than in nuclear DNA, and to increase dramatically with age (4,5).

1.2. Mitochondrial DNA Repair

After the discovery that there is no repair of UV-induced pyrimidine dimers in mitochondrial DNA (6), and in view of the high copy number, it was generally assumed that mitochondria lacked the capacity for DNA repair. Since then, however, several mammalian mitochondrial DNA repair enzymes have been detected, and many types of damage have been shown to be repaired (7–20). The gene-specific repair assay is ideal for studying mitochondrial repair, since it does not require the isolation of mitochondria, instead relying on sequence-specific probes of total DNA on a Southern blot (Fig. 2).

This chapter presents a method for applying the gene-specific repair assay to the measurement of 8-oxo-dG in mitochondrial DNA. To damage the DNA, cells are treated with photoactivated methylene blue (MB). MB is a cell-permeant cation, and is in equilibrium in the cells with the uncharged and colorless leucomethylene blue (21). When photoactivated, MB leads to DNA damage, with 8-oxo-dG being the major product of the reaction (22–24). When human fibroblasts are pretreated with MB and exposed to light, oxidative damage is induced in the mitochondrial DNA (25). To measure the damage, the bacterial repair enzyme formamidopyrimidine DNA glycosylase (Fpg) is used. Fpg recognizes oxidatively modified deoxyguanosine, particularly 8-oxo-dG, in double-stranded DNA, and excises it to generate a single-strand break (26,27).

1.3. Assay Development

As noted above, a limitation in the development of gene-specific assays for oxidative damage is the induction of the lesion of interest without the induction of other damage, which interferes with the measurement of repair. MB induces far more damage in mitochondrial DNA than in nuclear DNA, making it useful for repair measurements of 8-oxo-dG in mitochondrial DNA, but not in nuclear DNA. We have used another agent, acridine orange, to induce DNA damage in nuclear DNA with reasonable efficiency (28), but we have most extensively characterized MB as we developed methods to study mitochondrial repair. Other types of oxidative stress that can be considered are γ - and X-irradiation, as well as many other chemical agents that differ in their ability to penetrate the cell, their intracellular localization, and the chemical pathway by which the oxidative stress is generated. The following will describe assays to measure the repair of 8-oxo-dG in mitochondrial DNA, which will also serve as models in the development of new assays for measuring similar damage in nuclear DNA, or in the development of methods for measuring other lesions in both genomes (**Fig. 3**). Where possible, notes that will assist the investigator in using this protocol as a base on which to develop assays for other lesions, using other agents and other enzymes, have been included.

The methods presented below are grouped into several sections. **Subheadings 3.1.** and **3.2.** describe the preparation of DNA for use as a positive control and the optimization of reaction conditions to give complete cleavage at sites of damage while minimizing nonspecific cutting. These protocols will be of use if a new Fpg preparation of unknown specific activity or purity has been purchased, but also may serve as model protocols in the characterization of any repair enzyme for use in gene-specific repair experiments. **Subheading 3.3.** establishes the sensitivity of a previously untested cell line to photoactivated MB, both in terms of survival and of the levels of DNA damage that are

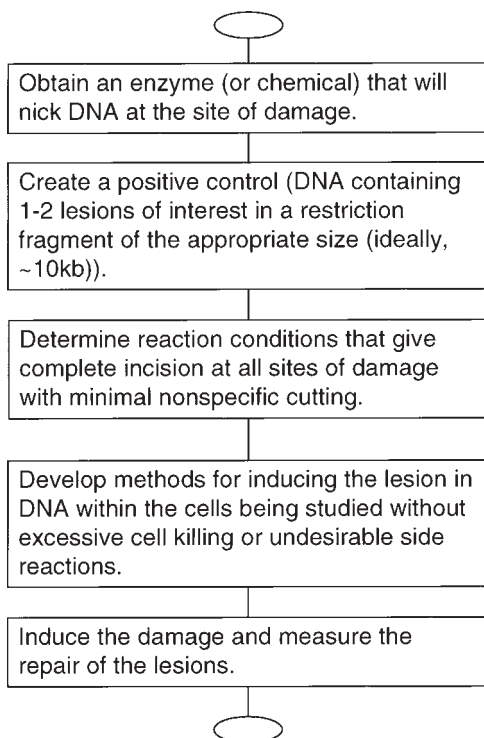


Fig. 3. A flow chart of the basic steps necessary to develop a gene-specific damage and repair assay for a new lesion.

induced. In addition, the protocols given may be used as models in testing other agents for the induction of DNA damage prior to gene-specific repair studies. **Subheading 3.4.** describes the actual repair assay in detail. **Subheading 3.5.** covers the essentials of data analysis, including theoretical considerations. Finally, **Subheading 3.6.** provides support protocols that are needed for the success of any gene-specific repair assay, regardless of the type of lesion.

2. Materials

2.1. Damaging Purified DNA In Vitro

1. Desk lamp with a 100-W tungsten bulb.
2. DNA, 200 $\mu\text{g/mL}$ in distilled H_2O .
3. Ice.
4. Methylene blue (MB), 100 μM (Ricca Chemical Company, Arlington, TX: $\epsilon = 89125$, $\lambda_{\text{max}} = 655 \text{ nm}$) (29).
5. Plastic tissue-culture dishes, six-well, clear (Corning #430343 or equivalent).
6. Polypropylene tubes, 15 mL.

7. TE: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
8. Ammonium acetate, 11 M.
9. Ethanol, 70 and 95% (or absolute).

2.2. Determination of Enzyme:DNA Ratios and Digestion Times

1. 10X Alkaline loading buffer: 25% Ficoll, 10 mM EDTA, 0.025% (w/v) bromocresol purple, 0.5 M NaOH (NaOH is not added until the day of use).
2. Damaged DNA in 1X reaction buffer: 9.5 µg of DNA in a 285-µL final volume.
3. Eppendorf tubes, 0.5-mL capacity.
4. Fpg stock: Fpg in sterile Fpg storage buffer: 50 mM HEPES-KOH, pH 7.5, 100 mM NaCl, 1 mM EDTA, 50% glycerol. Fpg is stable for at least a year at -20°C in this buffer. It is available from Trevigen, Inc., Gaithersburg, MD. (Inquire about pricing for large quantity purchases.)
5. 5X Reaction buffer (per mL; prepare just before use)
 - a. 500 µL of 10X Fpg reaction buffer (500 mM Tris-Cl, pH 7.5-8.0, 500 mM KCl, 10 mM EDTA: Sterile aliquots may be kept frozen for years).
 - b. 50 µL of 50 mg/mL bovine serum albumin (BSA) (Boehringer Mannheim, Indianapolis, IN).
 - c. 450 µL of dH₂O.
6. 1X Reaction buffer: Prepare by 1:5 dilution of 5X reaction buffer.
7. Undamaged DNA: 9.5 g of DNA in a 285 µL final volume.

2.3. Determining Conditions for Damage Induction in Untested Cell Lines

1. Tissue culture plates, 96-well (Costar 3596 or equivalent, Costar Corporation, Cambridge, MA).
2. DPBS medium (with calcium and magnesium) (Gibco BRL, Gaithersburg, MD).
3. DPBS medium (without calcium and magnesium) (Gibco BRL).
4. DPBS medium (without calcium and magnesium) (Gibco BRL) + 1% D-glucose. Filter sterilize using a 0.22-µm filter.
5. MB (*see Subheading 2.1., item 4*), 200 µM in DPBS (with calcium and magnesium) containing 1% D-glucose: filter sterilize using a 0.22-µm filter.
6. Methanol or Histochoice Tissue Fixative MB (Ameresco, Solon, OH).
7. Ethidium bromide solution, 20 µg/mL.
8. Fluorescence-capable plate reader, imaging system, or microscope.

2.4. Damage Induction and Repair Incubation

1. Cells in 15-cm tissue-culture plates (Falcon 3025 or equivalent, Becton Dickinson Labware, Lincoln Park, NJ).
2. DPBS (with calcium and magnesium).
3. DPBS (without calcium and magnesium).
4. MB in DPBS (with calcium and magnesium) containing 1% D-glucose. (Prepare at the concentration established in **Subheading 3.3.**) Filter-sterilize using a 0.22-µm filter.

5. Repair medium (normal growth medium): 5'-bromo-2'-deoxyuridine (Sigma, St. Louis, MO; $\lambda_{\max} = 278 \text{ nm}$, $\epsilon = 8820$) and 5'-fluoro-2'-deoxyuridine (Sigma, $\lambda_{\max} = 268 \text{ nm}$, $\epsilon = 7570$) may be added to final concentrations of 10 and 1 μM , respectively (see **Note 1**). Sterile 1000X stock solutions of these reagents (in water) may be stored frozen for extended periods of time. Spectrophotometrically determine the exact concentrations of each separately prior to mixing.

2.5. Cell Lysis and DNA Isolation

1. 10X proteinase K (Gibco BRL): 5 mg of proteinase K/mL of lysis buffer. Make immediately prior to use.
2. Cell scraper.
3. DNase-free RNase, 100 $\mu\text{g/mL}$.
4. Ice slurry.
5. Lysis buffer: 0.5 M Tris-HCl, pH 8.0, 20 mM EDTA, 10 mM NaCl, 1% sodium dodecyl sulfate (SDS). Stable for months at room temperature.
6. Saturated NaCl (approx 6 M).

2.6. Restriction Digestion

1. Minigel apparatus and supplies.
2. Restriction enzyme and 10X restriction buffer.
3. Sample DNA.

2.7. Purification of Parental DNA (CsCl Gradient and Fluorescent Localization)

1. Centricon 30 filtration tubes (Amicon, Inc., Beverly, MA).
2. Cesium chloride.
3. Coffee grinder (optional).
4. DNA standards (1, 10, 20, 40, and 80 ng/ μL) (see **Note 2**).
5. Ethidium bromide, 40 $\mu\text{g/mL}$.
6. Metal spatula (for stirring salt in ultracentrifuge tubes).
7. Mineral oil.
8. Restricted DNA.
9. Small beaker and foil.
10. Syringe and 18-gage needles.
11. Ultra-Clear 16 $\times$ 76 mm ultracentrifuge tubes with lids (Beckman, Palo Alto, CA; see **Note 3**).

2.8. Gel Electrophoresis

1. Agarose, molecular biology-grade.
2. Alkaline running buffer: 1 mM EDTA, 30 mM NaOH.
3. Gel apparatus with buffer recirculation system.
4. Lambda *Hind*III-digested marker DNA.
5. 500 mM Na-EDTA, pH 8.0.
6. 10 M Sodium hydroxide.

2.9. Southern Transfer

1. Blotting paper.
2. Ethidium bromide, 10 mg/mL.
3. Hybrisol (Oncor, Rockville, MD) (*see Note 4*).
4. 0.25 *M* Hydrochloric acid.
5. Neutral gel wash: 0.5 *M* Tris-HCl, pH 7.5, 1.5 *M* NaCl.
6. pH paper.
7. 1 *M* Sodium hydroxide.
8. 2X SSPE: 2.2 mM EDTA, 0.02 *M* sodium phosphate, pH 7.0, 0.36 *M* NaCl.
9. Transfer apparatus.
10. UV source, handheld, short range.

2.10. Hybridization

1. Blotting paper.
2. Hybrisol (Oncor, Rockville, MD) (*see Note 4*).
3. Labeled ribonucleotide triphosphate.
4. PES: 40 mM sodium phosphate, pH 7.2, 1% SDS, 1 mM EDTA.
5. PSE: 250 mM sodium phosphate, pH 7.2, 2% SDS, 1 mM EDTA.
6. Riboprobe template.
7. SP6/T7 Transcription kit (Boehringer Mannheim).
8. 2X SSPE: *see* item 8, **Subheading 2.9**.

3. Methods

3.1. Damaging Purified DNA In Vitro

1. Isolate and restrict the DNA as described in **Subheadings 3.6.1. to 3.6.3.** (*see Note 5*). Resuspend it in dH₂O at 200 µg/mL (*see Note 6*). Be certain to retain an equal amount for use as an undamaged (negative) control.
2. Prepare one foil-covered 15-mL polypropylene tube for each 200 µg of DNA to be treated. To each, add 400 µL of 11 *M* ammonium acetate.
3. Place a six-well dish on ice, and adjust a lamp so that a 100-W tungsten bulb is 18 cm from the dish (*see Note 7*).
4. Dilute 100 µM MB 1:25 with dH₂O. Use a foil-covered tube. (Make a volume equal to the volume of your DNA solution.)
5. **Turn out the white lights**, and then mix the DNA and MB solutions 1:1 (*see Note 8*).
6. Aliquot the MB:DNA mixture into a six-well dish, 2 mL/well.
7. Turn on the lamp for 5 min. (This will give 1–3 lesions/10 kb. *See Note 9.*) The lid may be on or off the dish as long as it is clear in the visible spectrum.
8. Transfer the DNA from the six-well dish to the prepared tubes containing ammonium acetate solution, 1 well/tube. Ethanol-precipitate the DNA. Continue to work in dim blue light until the pellet has been washed twice with 70% ethanol. Do not allow the pellet to dry: spin briefly a final time to bring all drops of ethanol to the bottom of the tube, and use a pipet to remove the last bit of ethanol.
9. Resuspend the DNA in 500 µL of TE/tube, quantitate, and dilute to 200 µg/mL.

3.2. Determination of Enzyme:DNA Ratios and Reaction Times (see Note 10)

1. Prepare Fpg by serial dilution into 1X reaction buffer, 45 μL are needed of each of the following concentrations:
 - a. 10 ng/ μL .
 - b. 1 ng/ μL .
 - c. 0.1 ng/ μL .
 - d. 0.01 ng/ μL .
2. Aliquot the damaged DNA, 15 μL /tube, into 18 of 36 Eppendorf tubes. Aliquot the undamaged DNA into the remaining 18 tubes.
3. To one of the Eppendorf tubes containing damaged DNA, add 5 μL of Fpg dilution A, repipeting to mix. Place in a 37°C water bath, and set a timer for 80 min (see Note 11). At 1 min intervals, repeat for Fpg dilution B, then for C, and so forth, and then for all four dilutions using undamaged DNA.
4. Repeat **step 3**, setting a timer for 40 min. Repeat again for 20 min and for 10 min.
5. Stop the reactions at the appropriate times by the addition of 2.2 μL of 10X alkaline loading buffer with finger-vortexing to mix. Add 5 μL of 1X reaction buffer and 2.2 μL of 10X alkaline loading buffer to the remaining tubes of DNA, which will not be treated with Fpg and will serve as the 0 time-point. Incubate all tubes at 37°C for 15 min to denature the DNA fully.
6. Centrifuge the samples briefly to bring condensation to the bottom of the tubes.
7. Run an alkaline gel and transfer for use in a Southern blot as described in **Subheadings 3.6.4. and 3.6.5.**, Probe for mitochondrial sequences (see **Subheading 3.6.6. and Note 12**).
8. Quantitate band volumes for the fragment of interest (in this case, the mitochondrial genome) in the Southern blot, and calculate the average number of enzyme incisions per band, as described in **Subheading 3.5**.
9. Mark as unusable any points for which the band intensity is too weak to quantitate accurately.
10. (a) Note the highest enzyme:DNA ratio at which little or no cutting was seen in control (undamaged) DNA after 20 min. This is the highest enzyme:DNA ratio that will be of use. (b) At each enzyme concentration and time-point, subtract the number of incisions observed in control (undamaged) DNA from the number seen for damaged (MB-treated) DNA. Plot “specific incisions” as a function of enzyme concentration for the 40 min time-point (see Note 13). Note the lowest ratio that gave the number of incisions corresponding to the number of lesions (that is, that gave complete cutting). It is unlikely that a lower ratio will be useful, but see Note 11.
11. Plot the number of incisions as a function of reaction time for the lowest enzyme:DNA ratio determined to be effective (in **step 10**, above). Note the time at which the reaction reached completion. The reaction can occur quickly and may be complete at the earliest time assayed.
12. Select the enzyme:DNA ratio and time to use in subsequent experiments based on the above data. Bear in mind that longer reaction times allow easier staggering

of reactions (starting tubes at, for example, 1-min intervals) and that it is best to allow a safety margin both in enzyme concentration and in reaction times, so that minor pipeting errors, errors in DNA quantitation, or fluctuations in pipeting times will not affect the outcome of the reaction. (That is, it will still go to completion, and nonspecific cutting will be absent.)

3.3. Determining Conditions for Damage Induction in Untested Cell Lines (see Note 14)

Plate cells in four 96-well dishes. The objective is to have the cells reach ~80% confluence approximately 24 h after plating. (For human fibroblasts, plate 5000 cells per well.) Leave column 12 as a blank (no cells).

3.3.1. Effect of MB Concentration on Cell Survival

1. Once the cells have reached 80% confluence, rinse two of the dishes twice with DPBS (+Ca,+Mg) and then change the medium, replacing it with 100 μ L of DPBS (+Ca,+Mg) containing 1% D-glucose. (Rapid changing can be accomplished by inverting the dish onto three folded paper towels that have been sprayed lightly with 70% alcohol to kill anything that might be on the top layer. The towels absorb the old medium. A multichannel pipet is then used to replace the medium.)
2. **TURN OFF THE WHITE LIGHTS: All subsequent steps must be performed under dim blue light (see Note 8).**
3. Add 100 μ L of 200 μ M MB to the first column. Repipet to mix, and then transfer 100 μ L to the next column. Repeat the serial dilution until column 10 is reached.
4. Return the cells to the 37°C incubator for 1 h.
5. After pretreatment is complete, rinse each plate twice with DPBS (–Ca,–Mg), leaving the second wash on the cells. Expose one dish to 4 min of light (see Notes 7 and 15), and then discard the final wash on both dishes.
6. Fix the cells in methanol or Histochoice Tissue Fixative for 10 min, and then allow to dry for several hours. Stain with 20 μ g/mL ethidium bromide (see Note 16), discard the excess, and read on a fluorescence plate reader, a fluorescence imaging system, or by counting several fields in a fluorescence microscope.
7. Subtract any background fluorescence (the column without cells), and express the fluorescence in each column as a percentage of the fluorescence seen in the MB-untreated column (i.e., column 11).
8. Two curves are obtained corresponding to cell attachment, with and without damage, immediately after treatment as a function of MB concentration.
9. Some cell lines are more sensitive than others to some types of damage. Note the concentration of MB that gives 50% survival after light exposure (as measured by attachment) for each plate.

3.3.2. Effect of Pre-exposure to MB

1. Dilute an appropriate amount of MB using DPBS (+Ca,+Mg) + 1% D-glucose. The final concentration should be the one just determined experimentally.

2. **Turn off the white lights: All subsequent steps must be performed under dim blue light** (*see Note 8*).
3. For the remaining two 96-well dishes, remove the medium from two columns, wash twice with DPBS (+Ca, +Mg), and replace with the MB. Set a timer for 2 h, and return the cells to the 37°C incubator. Every 30 min, repeat for two more columns of cells. (Three columns serve as controls, and the fourth is the cell-free blank.)
4. After pretreatment is complete, rinse each plate twice with DPBS (–Ca, –Mg), leaving the second wash on the cells. Expose one dish to 4 min of light. Discard the final wash.
5. Fix the cells in methanol or Histochoice Tissue Fixative for 10 min, and then allow to dry for several hours. Stain with 20 µg/mL ethidium bromide (*see Note 16*), discard the excess, and read on a fluorescence plate reader, a fluorescence imaging system, or by counting several fields in a fluorescence scope.
6. Subtract any background fluorescence (the column without cells), and express the fluorescence in each column as a percentage of the fluorescence seen in the MB-untreated column.
7. Two curves are obtained corresponding to cell attachment, with and without damage, immediately after treatment as a function of pre-exposure time.
8. Note the amount of variability with time. Ideally, minor variations in pretreatment time will not have an effect on the cellular response. If it does, however, this must be kept in mind so that pretreatment times are extremely precise, or else other pretreatment times must be tested.

3.3.3. Determining the Level of Initial Damage (*see Note 14*)

Plan an experimental timetable that will allow you to stagger the treatment of individual plates in a convenient manner as described in the following protocol, with duplicate plates receiving each of the following light exposures (in minutes): 0, 2, 4, and 6 (for a total of eight plates). In addition, three plates should serve as a full control (treated with neither MB nor light).

1. Plate the cells in 15 (or more, if a margin for error is desired) 15-cm tissue-culture plates (*see Note 17*). Allow to grow until approx 80% confluent.
2. For each plate, wash the cells twice with DPBS (+Ca, +Mg).
3. Add the MB solution (at the concentration determined in **Subheading 3.3.1.**). All cell treatments involving MB are performed under a dim blue safelight (*see Note 8*).
4. Return the cells to the incubator (for the amount of time established in **Subheading 3.3.2.**, above).
5. After the preincubation with MB is complete, wash the cells twice with DPBS (–Ca, –Mg). Leave the second wash on the cells.
6. Expose the cells to the appropriate amount of light (0, 2, 4, or 6 min).
7. Proceed to isolate the DNA and analyze the damage as described in **Subheading 3.6.**, below, combining the lysates from each time-point. **Protect the lysates from**

white light! The desired level of damage is 1–1.5 lesions/fragment (*see* **Notes 18 and 19**).

3.4. Damage Induction and Repair (see Note 20)

Plan an experimental timetable that will allow you to stagger the treatment of individual plates in a convenient manner. Each plate will have to be treated as described in the following protocol, with three or more plates used for each repair point. A duplicate determination (six plates in total) of the initial damage level is advised. Repair times can be varied: typical for 8-oxo-dG might be 0.5, 1, 2, 4, 8, and 24 h. In addition, three plates should serve as a negative control (and not be treated with MB or light), and three as an MB control (and not be treated with light). The latter serves to guard against accidental light exposure during DNA isolation.

1. Plate the cells in 15-cm tissue-culture plates. Allow the cells to grow until ~80% confluent.
2. For each plate, wash the cells twice with DPBS (+Ca,+Mg).
3. Add the MB solution.
4. Return the cells to the incubator (for the amount of time established in **Subheading 3.3.2.**, above).
5. After the preincubation with MB is complete, wash the cells twice with DPBS (–Ca, –Mg). Leave the second wash on the cells.
6. Expose the cells to the amount of light determined in **Subheading 3.3.3.**, above, to induce the correct number of lesions.
7. Remove the DPBS: (a) For the control cells and “0-min repair” time-point: proceed to isolate the DNA and analyze the damage as described below, combining the lysates from each time-point. **Protect the lysates from white light!** (b) For the cells that will be allowed to repair the damage: add 25 mL of repair medium, and return the cells to the incubator. At the appropriate time, lyse the cells as in (a), above.
8. Isolate and restrict the DNA as described in **Subheading 3.6.**, below (*see* **Note 1**).
9. Prepare Fpg by dilution into 1X reaction buffer. The final concentration should be based on the enzyme:DNA ratios determined in **Subheading 3.2.** to be optimal for the Fpg preparation being used. Five microliters are needed of each sample to be analyzed. (Remember to allow for the positive control, which is the purified DNA that was prepared in **Subheading 3.1.**).
10. Prepare 2.2 g of DNA in 33 μ L of 1X reaction buffer for each sample (*see* **Note 21**).
11. Aliquot 15 μ L into each of two 500- μ L Eppendorf tubes (*see* **Note 22**).
12. To one tube from each pair, add 5 μ L of the Fpg dilution, repipeting to mix. To the other, add 5 μ L of 1X reaction buffer. Place in a 37°C water bath, and set a timer for the reaction time determined earlier to be optimal in **Subheading 3.3.2.**
13. Repeat **step 12** for each sample, staggering the additions by at least 1 min so that reaction times are constant for all samples.

14. Stop the reactions at the appropriate times by the addition of 2.2 μL of 10X alkaline loading buffer with finger-vortexing to mix. Incubate all tubes at 37°C for 15 min to denature the DNA fully. At this time, an aliquot of *Hind*III-digested λ DNA mol-wt marker ($\sim 2\text{--}5\ \mu\text{g}$ for easy visualization on an alkaline gel and after Southern transfer) should be prepared and denatured in the same way.
15. Centrifuge the samples briefly to bring condensation to the bottom of the tube.
16. Run an alkaline gel as described in **Subheading 3.6.4**.
17. Transfer the gel for use in a Southern blot as described in **Subheading 3.6.5**.
18. Probe for mitochondrial sequences as described in **Subheading 3.6.6**.

3.5. Data Analysis

3.5.1. Signal Measurement

Quantify band volumes for the fragment of interest (in this case, the mitochondrial genome) in the Southern blot (*see* **Note 23**), and calculate the average number of enzyme incisions in each sample using the Poisson distribution as follows:

Incisions = $-\ln(BIE/BIC)$, where *BIC* refers to the band intensity in a “minus enzyme” lane, and *BIE* refers to the band intensity in an enzyme-treated lane (*see* **Note 24**). Mark as unusable any points for which “*BIE*” is too weak to quantify accurately.

3.5.2. Data and Error Analysis for an Experimental Series

1. If more than one gel was run from a single biological experiment, obtain a mean value for each time-point, and treat this as a single determination for purposes of error analysis.
2. Calculate the mean and standard error for the number of lesions at each time-point based on several biological experiments.
3. Comparison of two sets of repair curves: There are several methods for analyzing data resulting from the serial measurement of a process, such as DNA repair. The most commonly used is simply to treat each time-point as a separate measure, and compare the two curves one point at a time using a Student’s *t*-test. However, it is difficult to interpret the multiple (and far from independent) *P* values obtained. A much more acceptable approach is to evaluate the data using a multi-factor ANOVA (where the factors are the times allowed for repair, the individual experiments, and the comparison of biological interest).

Another, simpler alternative is to compare summary measures. Two that are especially relevant are the initial rate of repair and the maximum repair level attained.

The initial (absolute) rate of repair may be estimated as follows:

$$(D_0 - D_{t_1})/t_1 \quad (1)$$

where D_0 = initial damage, D_{t_1} = the damage at the first repair point, t_1 = time elapsed between damage and first repair measurement.

4. (a) When the results are presented graphically, repair is commonly expressed as a percentage rather than a raw lesion frequency. Percent repair is calculated as follows:

$$[(D_0 - D_t)/D_0]100 \quad (2)$$

where D_0 = initial damage and D_t = the damage at time t . (b) It is simplest to show the error in each time-point by reporting lesions/10 kb $\pm$ SEM in the text or a table prior to conversion to a percentage, since the number expressed as a percentage includes not only the error in the level of damage measured for each time-point, but also the error in the initial time-point and covariance owing to the influence of the initial level of damage on subsequent levels within a single experiment.

3.6. Support Protocols

3.6.1. Cell Lysis and DNA Isolation

1. After the cells have been washed with DPBS, remove the DPBS and add 2 mL of lysis buffer/15-cm dish. Wait at least 5 min. At this stage, the dishes may be kept for several hours at room temperature or at 37°C.
2. Use a cell scraper to assist in pouring the lysate into 15- or 50 mL conical polypropylene tubes. Protect from light by covering the tubes with aluminum foil if the DNA has been exposed to MB. When all the time-points have been collected, add 0.1 vol of 10X proteinase K, and incubate overnight at 37°C.
3. Add 1/4 vol of saturated NaCl solution to the warm lysate. Mix thoroughly and gently; heat briefly to 55°C if necessary to dissolve precipitate. Thorough mixing is necessary to prevent the formation of large SDS-protein complexes, which will trap high mol-wt DNA.
4. Cool, with swirling, by immersing the tube in an ice slurry. As the SDS precipitates, the protein that is bound to it does also.
5. Centrifuge for 30 min at 500g or greater.
6. Pour the supernatant (which contains DNA) into a fresh conical tube. Ethanol-precipitate the DNA (no extra salt is needed). Spin briefly a final time to bring all drops of ethanol to the bottom of the tube, and use a pipet to remove the last bit of ethanol. Resuspend the DNA in 0.1 mL of TE for each 1×10^6 cells.
7. Treat with 100 μ g/mL RNase A for 3 h at 37°C.
8. Ethanol-precipitate the DNA, again resuspending in 0.1 mL of TE for each 1×10^6 cells.
9. Quantitate the DNA (*see Note 17*).

3.6.2. Restriction Digestion

1. Select an appropriate restriction enzyme. (*PvuII*, for example, is useful to linearize human mitochondrial DNA.)
2. Restrict an aliquot of the DNA using ~ 10 U/ μ g of DNA for 3 h at 37°C. Save ~ 400 ng of unrestricted DNA for a test of the integrity of the DNA on a neutral agarose minigel. The integrity of the DNA reflects cell death, DNA damage, and

potential problems with the DNA isolation. The DNA concentration should be approx 200 µg/mL or less during the restriction, in the buffer recommended by the manufacturer of the restriction enzyme (see **Note 25**).

3. If the DNA is not fully restricted prior to proceeding with the experiment, the results will not be usable. It is worthwhile at this stage, therefore, to check the restriction using a small, native 0.5% agarose gel. At this time, also run 200 ng/lane of the unrestricted DNA. A tight band should be observed if the DNA has not degraded. The restricted DNA should be of lower molecular weight than the unrestricted DNA. If necessary, dilute the DNA further, and repeat the restriction.

3.6.3. Purification of Parental DNA:

*CsCl Gradient and Fluorescent Localization (see **Notes 1 and 3**)*

1. Weigh 6 g of CsCl into each tube. (Use the coffee grinder if the CsCl has clumped: this will speed step 3.)
2. Place the tube in a small beaker containing aluminum foil to hold the tube in an upright position. Tare the tube holder and the tube containing the CsCl on a balance.
3. Add the restricted sample to the centrifuge tube, and then add TE until the total liquid added weighs 4.70 g. Stir with a metal spatula until the CsCl is in solution.
4. Tare the empty tube holder.
5. Place the tube containing the sample DNA and CsCl in the holder and a top next to it. Add mineral oil to reach about 4 mm from the top. Note the weight (it will be ~22–22.3 g).
6. Place the cap assembly on the tube, making sure that it snaps into the bottom position. Tighten the hex nut.
7. Repeat **steps 5 and 6** with the other tubes, but add oil by weight so that all of the tubes are within 0.01 g of one another. Pair the tubes so that they are as closely matched by weight as possible. It may be necessary to add a tiny amount of oil with a syringe at this time to one or two of the tubes.
8. Place a screw into the top of each cap assembly.
9. Spin at 37,000 rpm in a Beckman Ti-50 rotor (124,000g) at 25°C for 36 h. Make sure that the deceleration is gradual (~6 min from 500 rpm (~23g) to 0).
10. Fasten the tube to a stand. Use a syringe filled with air to puncture the top of the tube, being careful not to disturb the gradient. Place fraction collection tubes under the gradient (1.5-mL Eppendorf tubes are most convenient). Use a small-gauge needle to puncture the bottom of the tube, and collect ~400 µL fractions by dripping the gradient from the bottom of the tube. Repeat until all samples have been fractionated.
11. Quantitate the DNA in each fraction using fluorescence: mix 2.5 µL of each fraction with 2.5 µL of 40 µg/mL ethidium bromide (see **Note 16**) on plastic wrap. At the same time, use 1, 10, 20, 40 and 80 ng of DNA (see **Note 2**) to create a standard curve. Photograph the fluorescence on a UV transilluminator. Use densitometry to quantify the dots on the negative (see **Note 26**).

12. Combine the appropriate fractions (those in the peak nearest the top of the centrifuge tube), and remove CsCl by using Centricon 30 tubes (or equivalent) (*see Note 27*).

3.6.4. Gel Electrophoresis

1. For a 20-kb gene of interest, use a 0.6% (w/v) gel, and increase it by approx 0.2% for each 5-kb decrease in fragment length. (Thus, for the linearized mammalian mitochondrial genome, a 0.75% gel is used.) Prepare the alkaline agarose gel by mixing the agarose, water, and EDTA (final concentration, 1 mM). Dissolve the agarose in a microwave, and then equilibrate it in a 55°C water bath for ~1 h. Add 10 M NaOH to a 30 mM final concentration (*see Note 28*), and then pour it immediately into the gel tray, preferably in a cold room. Check that no bubbles have formed on the gel comb (*see Note 29*).
2. Place the gel in the bed, fill the bed with running buffer, and prepare the system for buffer recirculation (*see Note 30*).
3. Prior to running the gel, prepare the required amount of 10X alkaline loading buffer. In addition, prepare enough 1X loading buffer to allow loading 20 µL/well.
4. Preload the gel using the 1X alkaline loading buffer. Let it sit in the wells for ~1 min, and inspect for leaks. Note which wells are unusable, if any. Prerun the gel at ~1.5 V/cm for 1 h to clear the wells.
5. Load the samples, being extremely careful to load quantitatively. Gel-loading pipet tips can be very useful for this purpose.
6. Perform electrophoresis overnight (assuming a 25 cm gel) at 1.5 V/cm with buffer recirculation.

3.6.5. Southern Transfer

1. Following electrophoresis, wash the gel at room temperature for 45 min in neutral gel wash.
2. Stain for 45 min in 1 µg/mL ethidium bromide (*see Note 16*), then destain in dH₂O for 30 min and photograph the gel.
3. Transfer the gel to 0.25 N HCl. Let sit for 30 min.
4. Transfer to 1 N NaOH. Let sit for 45 min. The gel is now ready for blotting.
5. Rapid transfer can be achieved with a Posiblot apparatus (Stratagene, La Jolla, CA), following the manufacturer's directions. Traditional overnight methods are also acceptable.
6. Following transfer, rinse the membrane several times with 2X SSPE. Using pH paper, check the pH of the SSPE wash: the procedure is complete when it remains at pH 7.0.
7. Pat the membrane dry between two pieces of blotting paper, then bake at 80°C under vacuum for 2 h. This allows visualization of the DNA on the blot by UV shadowing using a handheld short-range UV source. Use a No. 2 pencil to label the membrane and to note the position of the mol-wt markers.
8. Prehybridize overnight or longer in Hybrisol (*see Note 4*).

3.6.6. Hybridization

1. Prepare a riboprobe against the sequence of interest (*see* **Notes 31** and **32**) using a kit and following the manufacturer's instructions.
2. Hybridize overnight in Hybrisol at 45°C (*see* **Note 4**).
3. After hybridization, wash twice with 2X SSPE at room temperature to remove unbound probe.
4. Wash twice with PSE at 65°C (or lower, depending on the probe) and then once with PES at 65°C (or lower, depending on the probe) to remove nonspecifically bound probe.
5. Use blotting paper to blot the membrane dry lightly, and then place in plastic wrap (*see* **Note 33**) and expose to X-ray film (or place in a PhosphorImager cassette) (*see* **Note 23**).

4. Notes

1. CsCl centrifugation to separate parental and daughter DNA may be necessary if cell replication or mitochondrial replication in the absence of cell replication is able to occur during the repair period. (In at least some cell lines, MB prevents both.) It requires that 5'-bromo-2'-deoxyuridine and 5'-fluoro-2'-deoxyuridine be included in the repair medium, so that parental and daughter DNA differ in density. It should also be noted that variations in GC/AT content can cause some sequences (for example, the nuclear ribosomal sequences) to move away from the bulk of the DNA in the gradient. The mobility of specific sequences can be monitored by use of dot or slot blots. (Also, since mitochondrial DNA can replicate independently of the nuclear DNA, dot or slot blots are necessary if visualization of parental and daughter mitochondrial DNA peaks is desired.)
2. DNA standards can be prepared from any high-mol-wt DNA by serial dilution of DNA that has been extensively purified and quantitated by UV absorption.
3. This protocol is written for a Beckman ultracentrifuge, and may require modification if a different ultracentrifuge is used.
4. Other hybridization solutions will also work.
5. (a) If a new lesion or new enzyme is being characterized, it is useful to work with plasmids at this stage, rather than total mammalian DNA. Transitions from supercoiled to nicked circle to linear can easily be monitored in neutral gels with ethidium fluorescence, rather than the more laborious Southern blot methods required for specific sequences in total DNA. This also avoids concerns about the stability of the lesion in the alkaline conditions used to denature DNA for the Southern blot method. (b) If a new lesion is being characterized, its stability under the conditions used to denature the DNA for the overnight gel must be ascertained (unless this is known from the literature). Identical aliquots of damaged plasmid are loaded onto neutral and denaturing gels both with and without prior treatment with the repair enzyme. The percent remaining supercoiled is determined and compared between the two gels. Values should be determined in duplicate or triplicate for precision. (Since ethidium bromide does not efficiently bind denatured DNA, the use of a Southern blot is advised.) Nonalkaline-based

methods for denaturing DNA are also available (glyoxal). Here too, however, a test of lesion stability would be required.

6. The protocol is written for 400 μg , but more or less DNA can be treated depending on the number of experiments planned. Five to 10 μg will be used on each repair gel as a positive control, and an additional $\sim 20 \mu\text{g}$ will be used to characterize a new preparation of enzyme.
7. The light exposure, if measured, should be 50–70 kJ/m^2 . (Measurement may be made with an IL1400A Radiometer/Photometer [International Light, Inc., Newburyport, MA]). Owing to the intensity of the light, light must be measured through a screen that reduces the intensity by a known amount.)
8. Work with MB should be done in a dim blue light. Safelights for photosensitive agents can generally be made with inexpensive plastic available from a local industrial plastic supplier. Obtain small samples of approximately the same color as the reagent, and check the spectrum on a scanning spectrometer. Choose the one that most closely matches the reagent to construct the safelight.
9. The goal is to obtain 2–3 lesions/10 kb of double-stranded DNA (dsDNA). If there is an independent method to measure this level, it is recommended that it be used. Otherwise, the enzyme itself must be used, which may require one to two iterations. That is, a rough estimate of optimal enzyme conditions is used to quantify damage in a DNA sample. This is used to select the sample with 2–3 lesions/10 kb. This sample is then used to refine further the optimal enzyme conditions.
10. This is suggested for each new batch of enzyme. At the very least, a new batch of enzyme should be directly compared to an old batch using both undamaged and damaged DNA. It is critical to test each new preparation, and to work in conditions where cutting is complete and nonspecific cutting is not extensive.
11. Eighty minutes is arbitrarily the longest time-point. It is possible that a longer incubation, perhaps even overnight, could be used with lower enzyme concentrations to conserve enzyme.
12. It is useful to optimize the assay conditions by probing for mitochondrial DNA for two reasons: one, the genome is completely sequenced, and two, it is multicopy, so sensitivity is increased (and less DNA is needed per lane, thereby saving on enzyme costs). Remember that assay sensitivity is optimal between 1 and 2 lesions/fragment, so if another gene is to be assayed, the restriction of the mitochondrial genome and initial damage levels should be adjusted to approximate the desired conditions. Endogenous damage has not been seen in the mitochondrial DNA of cultured cells (25,30), and is thus not a confounding factor.
13. A clear plateau should be seen. If it is not seen, the reaction is not going to completion. The number of incisions seen at the plateau corresponds to the actual number of lesions induced in the damaged DNA.
14. Three points should be considered.
 - a. Each time a new cell type is to be treated, it is advisable to test its sensitivity to the agent being used. In addition, the cells should be as close as possible in terms of growth state and culture conditions to the conditions that will be used in the actual experiment.

- b. Differences in initial damage levels are a confounding variable when two treatment populations are to be compared. If, for example, the time required for a given level of repair is twice as great in one population as in another, this could be explained by a twofold difference in the number of lesions removed per minute, or by a twofold difference in the initial level of damage. It is therefore important to establish conditions that yield similar initial lesion frequencies if two populations are to be compared.
 - c. Differences in initial lesion frequency may be unavoidable in a single treatment population. For example, there are 2.38-fold more guanines in the heavy mitochondrial strand than in the light strand. Randomly induced damage to guanine will, therefore, result in more initial damage in the heavy strand than in the light strand. In this case, it is biologically relevant to treat the two strands as a single system, ignoring the difference in initial lesion frequency. If the probability of any lesion being repaired is equal and not a function of strand placement, the fraction of initial lesions should be the same in each strand at any given time in the repair process. If, on the other hand, it is not random, the fraction remaining should depend on which strand is being examined.
15. The light source for all experiments is, ideally, a 100-W tungsten bulb situated beneath the plate providing 18.5 mW/cm^2 of visible light. A filter may be necessary to provide even intensity across the dish. To prevent heating, the tissue-culture plates should be separated from the light source by a ventilated chamber. Exposure from above is also possible, using a conventional desk lamp; in this case, an orbital shaker at low speed can be used to move the plate in such a way that light exposure is not higher in the center of the plate than at the periphery, and a small fan can be used to blow air across the plate to prevent heat buildup (keeping the dish closed to avoid contamination).
 16. Ethidium bromide is a carcinogen. Handle with care, and dispose of waste in an appropriate manner.
 17. The expected DNA yield is $\sim 7\text{--}10 \text{ }\mu\text{g}/1 \times 10^6 \text{ cells}$. DNA can be quantitated using UV spectroscopy, or, to conserve DNA, fluorescent quantitation using a kit, such as the Picogreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR), is also effective. (The cell-culture conditions suggested in these protocols should yield enough DNA for analysis of single-copy genes. If only multicopy genes, such as mitochondrial DNA, are to be assayed, smaller plates and fewer cells may be used.)
 18. Cell death prior to the measurement of initial damage does not affect the repair determination. Cell death subsequent to the initial measurement becomes a problem only if the dying cells had a higher level of initial damage than the surviving cells, which would lead to an overestimation of the extent of repair. Nevertheless, it is best to avoid cell death if possible, and if DNA damage is sufficient (1 lesion/restriction fragment of interest) at levels that allow all cells to survive, that is the dose that should be used. If, on the other hand, a higher dose must be used, one option is to limit repair measurements to earlier times, before death or detachment occur.

19. A final series of survival studies, done at the selected MB concentration, pre-exposure time, and light exposure is advisable, this time measuring attachment during the repair period also. Ideally, little or no cell death will occur after damage, during the repair period. If it does, it is usually assumed that cell death occurred randomly, rather than that a subset of cells received a greater amount of initial damage, unless there is reason to think otherwise.
20. (a) When two cell lines are compared, ideally they will have completed a similar percentage of their in vitro life-span. This can sometimes be arranged for mortal cell lines simply by growing the “younger” line an extra passage or two. For this reason, for mortal cell lines, the replicative status of the cells should be assayed by determining the “30-h labeling index” prior to the repair experiment. The 30-h labeling index is the fraction of cells that incorporate labeled nucleotides into their DNA in a 30-h incubation. This can be done either nonisotopically using a kit, such as the BrdU Labeling and Detection Kit (Boehringer Mannheim), or by tritium incorporation. Data analysis: $\log(\text{percent nondividing cells})$ is proportional to the completed life-span (31,32).
(b) No two cell lines, even sister clones, have exactly the same maximum population doubling level (PDL). PDL refers to the population doubling level of cells in culture. It is a more objective measure of cell growth than passage number, since the latter does not give any information on the split (e.g., 1:2 or 1:4) that was used at each passage. In this context, it is worth noting that immortalized cell lines may show genetic drift, so that after many cell divisions, they may not be identical to the parent line or to cells of earlier passage. Calculate the PDL of the culture. The formula for the change in PDL from one passage to the next is: $\log(\text{final cell number}/\text{initial cell number})/\log(2)$. If the cell line being tested has been transfected, calculate the posttransfection PDL.
21. The DNA concentration would be increased 5- to 10-fold if single-copy genes were to be studied, rather than the multicopy mitochondrial genome.
22. It is critical for the success of the assay that the two tubes contain exactly the same amount of DNA.
23. These protocols absolutely require a method for quantitating the relative signal from the bands. A system, such as the PhosphorImager (Molecular Dynamics, Inc. Sunnyvale, CA), is ideal owing to its extended linear range. However, photographic film and densitometry may also be used if care is taken to remain within the linear range of the film.
24. The sensitivity of the assay is limited at both extremely high lesion frequencies (which cause weak, poorly quantifiable bands in the “minus enzyme” lanes) and at extremely low lesion frequencies (in which case the “minus” and “plus” enzyme lanes both give approximately the same signal, and their ratio is thus greatly altered by even slight differences in loading or transfer).
25. DNA that is not fully dissolved will not restrict.
26. If exact quantitation is not desired, and only the location of the parental and daughter peaks is sought, then densitometry is not necessary. The peaks will be clearly visible.

27. CsCl may also be removed by dialysis if preferred.
28. The agarose will caramelize (turn brown) if it is too hot when the NaOH is added.
29. Since bubble formation is a problem at the cooler gel-pouring temperatures used for alkaline agarose gels, a gravy pitcher, purchased from a local grocery store, can be used to prevent bubbles from reaching the gel tray.
30. Buffer recirculation is necessary with alkaline gels. This can be accomplished in two ways: over the gel (in which case, run the gel long enough for the DNA to enter the agarose prior to beginning the recirculation), or under it. For the latter, have the gel tray sitting on a spacer, and cover the top of the gel with glass (or heavy plastic, such as Plexiglas). The latter method has two advantages: diffusion of smaller DNA fragments and marker dyes is minimized, and the recirculation can be begun as soon as the gel is loaded.
31. The use of riboprobes is recommended for two reasons: strand-specific probing is possible, and stripping the membrane without any residual probe remaining is easily accomplished. (A room temperature wash in 0.4 M NaOH for 1 h, followed by one or two neutralizing washes containing 0.5% SDS, is usually sufficient.)
32. The riboprobe template plasmids used by our laboratory were prepared by cloning mitochondrial sequences amplified by PCR into the PCR II vector (Invitrogen, Carlsbad, CA) between the T7 and SP6 promoter sequences. The sequences were from 652–3226 and from 5905–7433 of the mitochondrial genome (numbering based on the Anderson sequence [33]).
33. The plastic must be wrinkle-free, front and back, for accurate quantitation. Minor variations in the distance between the blot and the film (or PhosphorImager screen) will alter the signal intensity significantly. Clear plastic page protectors are not subject to wrinkling and can be substituted for plastic wrap.

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Characterization of DNA Strand Cleavage by Enzymes That Act at Abasic Sites in DNA

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1. Introduction

Apurinic/apyrimidinic (AP) sites are one of the more common lesions formed in DNA that, if left unrepaired, can represent potential sites of mutation (1). To illustrate the importance of these sites, all organisms that have thus far been tested have been found to contain enzymes that incise abasic sites to initiate the repair process. Generally, these enzymes fall into two categories. The predominate AP endonuclease, at least quantitatively, is one that hydrolytically cleaves 5' and adjacent to an AP site (Fig. 1), producing nucleotide-3'-hydroxyl and 5'-deoxyribose-5-phosphate termini (2). The other class of enzyme that acts at AP sites is part of a base excision repair pathway that is initiated by an *N*-glycosylase activity directed toward a modified or nonconventional base in DNA. Some of these *N*-glycosylases also possess AP lyase activity that cleaves DNA 3' to an AP site via a β -elimination reaction to leave a 3' 4-hydroxy-2-pentenal-5-phosphate (Fig. 1). An example of this is endonuclease III in *Escherichia coli*, which is a broad specificity enzyme for the repair of oxidative damage to DNA. Other *N*-glycosylase/AP lyase repair proteins not only cleave 3' to an AP site, but follow this with a δ -elimination reaction to remove the AP site and leave 3'- and 5'-phosphate termini (Fig. 1). An example of this type of enzyme is the *E. coli* Formamidopyrimidine glycosylase (Fpg), which catalyzes a concerted β , δ -elimination reaction (3). Another example is *Drosophila* S3, but in this case, after catalyzing a β -elimination reaction, it appears as if the S3 protein dissociates from the AP substrate, and on a second encounter with the substrate, catalyzes a δ -elimination reaction (4).

We have previously employed three different abasic DNA substrates to characterize the type of strand cleavage carried out by various AP endonucleases

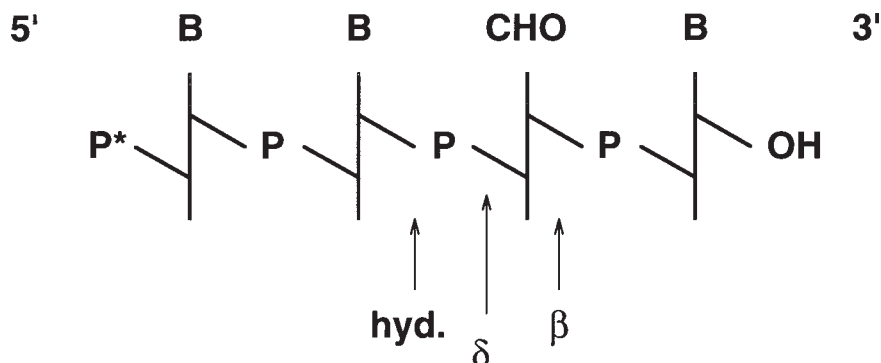


Fig. 1. Cleavage sites for AP endonucleases/lyases. The 5'-end is labeled P*, CHO represents the AP site that is cleaved either hydrolytically (hyd) or by a β -elimination reaction, and in some cases, by an additional δ -elimination. For convenience only one strand of the duplex is depicted.

and AP lyases present in *Drosophila* (4–6). One of these will be described here and utilizes a 5'-end-labeled DNA duplex oligonucleotide containing a single abasic site. After reaction with an AP endonuclease or AP lyase, the products of the reaction are separated on a polyacrylamide gel. Based on the migration of cleaved product, one can then easily visualize by autoradiography the type of strand cleavage possessed by the individual repair enzymes. Quantitation of the formed products can either be by video densitometric analysis of autoradiograms, or by Phosphorimager analysis and scanning of dried gels. We prefer this labeled oligonucleotide procedure over the others we have previously employed, because it yields a visual image that can easily convey to an audience the differences between the types of cleavage events possessed by different DNA repair proteins that have been highly purified. However, for less-pure enzyme preparations, the reader is encouraged to make assessments using a substrate developed by Levin and Demple (7) that can also distinguish between cleavage events carried out by AP lyases or AP endonucleases.

For the assay described here, our laboratory uses an oligonucleotide that is 37 bp in length (37-mer). Within the 37-mer is a single uracil residue that is placed at position 21 during the synthesis of the oligonucleotide. After 5'-end-labeling and gel purification of the single-stranded uracil-containing oligonucleotide, the complementary strand is annealed to create a duplex 37-mer. This forms a substrate for uracil-DNA glycosylase (8), which liberates the nonconventional base and forms in its place an abasic site (AP-37-mer).

Once the AP-37-mer is prepared, it can then be employed as a substrate for DNA repair proteins suspected to act on AP sites. Generally, this calls for enzymes that have been purified in order to lack the numerous nonspecific

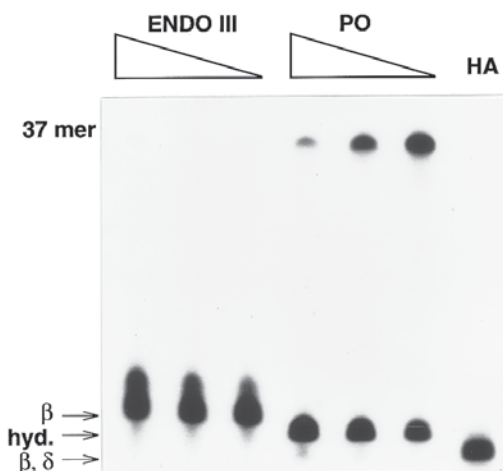


Fig. 2. Activity on AP-37-mer. Incubations with *E. coli* endonuclease III contained total protein amounts of 100, 200, and 400 pg. For *Drosophila* PO, incubations contained 50, 100, and 200 ng. HA, hot piperidine to generate a β , δ -elimination product.

nucleases present in crude protein extracts. Less than homogeneous preparations can on occasion be used if incubations are conducted in the presence of 10 mM EDTA that inhibits many nonspecific nucleases. The disadvantage of this is that many AP endonucleases are dependent on the presence of $MgCl_2$.

The DNA cleavage products of three different enzymes that act on abasic sites in DNA are presented in **Figs. 2** and **3**, and reveal how the individual migration products provide direct information on the type of DNA termini produced by each enzyme. Also presented is the electrophoretic mobility of the products of hot alkali (HA) treatment of the AP-37-mer, which is known to generate products with both 5'- and 3'-phosphoryl groups by a β , δ -elimination reaction at the AP site (**Fig. 2**, HA lane; **ref. 2**). DNA fragments containing a terminal phosphoryl group migrate faster than those of the same length, but lacking a terminal phosphate. Thus, HA treatment should present a landmark for those enzymes that generate a δ -elimination product, such as S3 (**Fig. 3**). It should also be noted that the product for S3 increases with increasing amounts of protein. This is believed to be because once S3 catalyzes a β -elimination (note in **Fig. 3** the same electrophoretic mobilities for the top band for S3 and *E. coli* endonuclease III, a known β -elimination catalyst [9]), S3 then dissociates from the substrate, and on a second encounter, catalyzes a δ -elimination reaction. This is in contrast to *E. coli* Fpg, which catalyzes a concerted β , δ -elimination reaction in which equal amounts of the β and δ product are revealed, regardless of time of incubation or amount of protein added to the reaction.

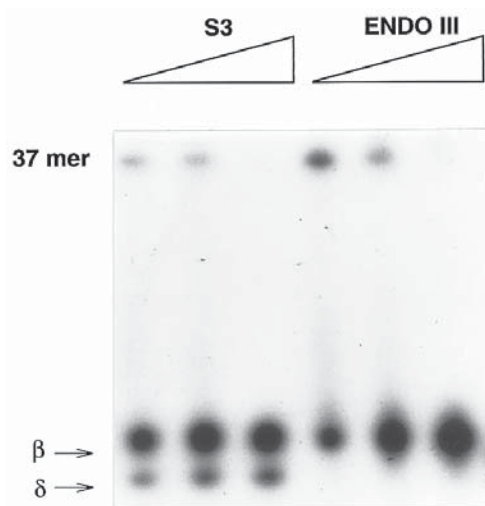


Fig. 3. Activity of *Drosophila* S3 (20, 40, and 80 pg) and *E. coli* endonuclease III (20, 40, and 80 pg) on AP-37-mer.

Results are also presented for *Drosophila* PO, which contains AP endonuclease activity and has previously been shown to cleave hydrolytically DNA 5' to an AP site (10). Since this activity does not leave a 3'-phosphoryl group, it will migrate slower than HA, but faster than a β -elimination product, since it is shorter in length when measured from the labeled 5'-end (Fig. 2).

2. Materials

All solutions should be made using molecular biology-grade reagents and sterile distilled water.

2.1. 5'-End-Labeling and Purification of Oligonucleotides Containing a Single Uracil Residue

The oligonucleotides used in our studies are commercially prepared to our specifications. The single-stranded oligos are deprotected and purified by spin-column chromatography (Gibco BRL). The individual single-stranded and purified oligonucleotides are then resuspended in distilled water to 10 pmol/ μ L.

1. T4 polynucleotide kinase, 10 U/ μ L (Stratagene, La Jolla, CA).
2. 10X T4 polynucleotide kinase buffer: 700 mM Tris-HCl, pH 7.6, 100 mM MgCl₂, 50 mM dithiothreitol (DTT), 1 mM spermidine-HCl.
3. γ ³²PATP, 10 mCi/mL, 6000 Ci/mmol (Amersham, Arlington Heights, IL).
4. 10X Annealing buffer: 100 mM Tris-HCl, pH 7.6, 100 mM MgCl₂, 10 mM EDTA.
5. Loading buffer: 50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol.

6. Phenol, molecular biology-grade, neutralized, and equilibrated with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
7. Phenol/chloroform/isoamyl alcohol mixture (25:24:1 by vol).
8. 40% Acrylamide stock: 38:2 acrylamide:*bis*-acrylamide in 100 mL of distilled water.
9. 10X TBE: 890 mM Tris-borate, 20 mM EDTA, pH 8.0.
10. Nondenaturing 20% polyacrylamide gel (per 100 mL): 50 mL of 40% acrylamide stock, 10 mL of 10X TBE, 500 μ L of 10% ammonium persulfate, 60 μ L of TEMED, and distilled H₂O to 100 mL final volume.
11. Centrex MF-0.4 microcentrifuge tubes (Schleicher & Schuell).
12. 35 mM HEPES-KOH, pH 7.4.

2.2. Cleavage of Uracil and Purification of the Duplex Oligo-Containing Abasic Site

1. Uracil-DNA glycosylase (GST-UDG fusion protein prepared in our lab; *see* **Note 1**).
2. 10X Uracil-DNA glycosylase buffer: 200 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10 mM DTT, and 100 μ g/mL bovine serum albumen (BSA).
3. 10 mM HEPES-KOH, pH 7.4.

2.3. Enzymatic Reactions and Electrophoresis

1. Abasic oligonucleotide (prepared as described in **Subheadings 3.1.–3.3.**).
2. Purified putative or known AP endonuclease/AP lyase (e.g., *Drosophila* S3 or PO).
3. 10X *Drosophila* S3 buffer: 300 mM HEPES, pH 7.4, 500 mM KCl, 10 μ g/mL BSA, 0.5% Triton X-100, 10 mM DTT, and 5 mM EDTA.
4. 10X *E. coli* Endo III buffer: 150 mM KH₂PO₄, pH 6.8, 100 mM EDTA, 100 mM β -mercaptoethanol, and 400 mM KCl.
5. 10X *Drosophila* PO buffer: 300 mM HEPES, pH 7.4, 1 M NaCl, 50 mM MgCl₂, and 500 mM KCl.
6. 1 M Piperidine.
7. Denaturing polyacrylamide gel: 16% polyacrylamide solution, 7 M urea, 1X TBE.
8. Formamide loading buffer: 96% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue, 10 mM EDTA.
9. 15% methanol: 10% acetic acid solution.
10. Whatman 3MM paper.
11. X-ray film (Kodak XAR-5) or Phosphorimager.

3. Methods

Characterization of endonucleases that act at abasic sites can be divided into several parts: First, a 5'-radiolabeled synthetic oligonucleotide containing a single deoxyuridine residue is prepared. This is then annealed to its non-radioactive complementary oligonucleotide. Once the duplex is purified, the uracil residue is removed by reaction with uracil-DNA glycosylase to form an AP oligo, which is then employed as a substrate for enzymes suspected to act on abasic sites. The reaction products are then separated on a DNA sequencing gel.

3.1. 5' End-Labeling and Purification of Oligonucleotides Containing a Single Uracil Residue

Bacteriophage T4 polynucleotide kinase is used to catalyze the transfer of the γ phosphate of ATP to the 5'-hydroxyl terminus of the uracil oligonucleotide. The following procedure produces sufficient quantities of 5'-end-labeled duplex oligonucleotides for several enzymatic reactions.

1. Prepare 5'-labeling reactions mixture in a 0.5-mL microcentrifuge tube containing the following:
 - a. ^{32} PATP 3 μ L
 - b. 10X kinase buffer 4 μ L
 - c. Oligonucleotide containing uracil 2 μ L
 - d. 2 U of T4 polynucleotide kinase 2 μ L
 - e. Distilled water to 40 μ L final volume
2. Incubate the reaction mixture for 30 min at 37°C.
3. Extract the reaction mixture once with phenol/chloroform/isoamyl alcohol.
4. Mix for 1 min, and then centrifuge at 12,000g for 3 min at room temperature in a microcentrifuge. Transfer the aqueous supernatant to a new tube. Add 2½ vol of ethanol, mix, and store the tube at -20°C for 1 h.
5. Recover the oligos by centrifugation at 12,000g for 15 min at 4°C in a microcentrifuge. Remove the supernatant, and leave the tube open at room temperature until all the ethanol has evaporated.
6. Dissolve the pellet in 20 μ L of distilled water.

3.2. Annealing Reaction (see Note 2)

1. Mix together in a microcentrifuge tube the following:
 - a. Labeled uracil oligos (1 pmol/ μ L) 20 μ L
 - b. Complementary strand (10 pmol/ μ L) 4 μ L
 - c. 10X annealing buffer 4 μ L
 - d. Distilled water to 40 μ L final volume
2. Incubate the annealing mixture at 75°C for 10 min.
3. Slowly cool to room temperature.
4. Add 10 μ L of loading buffer and mix well.
5. Separate the labeled duplex oligonucleotides on a 20% nondenaturing polyacrylamide gel and then subject to autoradiography.
6. Excise the band corresponding to the labeled duplex oligos from the gel, and transfer to a Centrex MF-0.4 microcentrifuge tube.
7. Crush the acrylamide gel into small pieces against the wall of the tube, add 200 μ L of 35 mM HEPES-KOH, pH 7.4, to the tube, and incubate for 5 h to overnight at 4°C to elute the labeled oligos from the gel (typically >95% of labeled oligonucleotide is eluted).
8. Collect the duplex oligos by centrifugation at 4000g for 3 min in a microcentrifuge.

3.3. Uracil Excision

1. Uracil-DNA glycosylases are used to hydrolyze the *N*-glycosidic bond between the deoxyribose sugar and uracil base. Reaction mixtures are as follows:

- a. Labeled uracil-containing duplex oligo 10 pmol
 - b. 10X uracil-DNA glycosylase buffer 4 μ L
 - c. Uracil-DNA glycosylase (1 U/ μ L) 1 μ L
 - d. Distilled H₂O to 40 μ L final volume
2. Incubate the reaction for 20 min at 37°C. Extract the reaction mixture once with phenol:chloroform and precipitate the DNA as described in **steps 3–5 of Subheading 3.1.**
 3. Dissolve the purified labeled duplex oligonucleotide in 10 mM HEPES-KOH, pH 7.4. It can be stored at 4°C for up to a week.

3.4. Enzymatic Reactions

1. Mix together in a microcentrifuge tube: ~1 pmol of γ -³²P abasic oligonucleotide (typically 10,000 cpm)

10X reaction buffer	1 μ L
Enzyme	x μ L
Distilled H ₂ O	to 10 μ L final volume

Incubate at 37°C for the desired time.
2. Stop the reactions by heating at 75°C for 10 min. Add 2 μ L of formamide loading buffer, heat for 4 min at 90°C, cool on ice, and then load immediately on a denaturing polyacrylamide gel.

3.5. Hot Piperidine Treatment

To 10 μ L of 5'-end-labeled abasic oligonucleotide, add 90 μ L of 1 M piperidine and incubate 30 min at 90°C. Lyophilize to dryness using a Speed Vac, and redissolve the pellet in 20 μ L of distilled H₂O, and repeat the lyophilization step twice more in order to remove all of the piperidine. Dissolve the remaining pellet in 50 μ L of formamide loading buffer, heat for 4 min at 90°C, cool on ice, and then load immediately on a denaturing polyacrylamide gel.

3.6. Analysis of Cleavage Activity by Denaturing Gels

Load an equal amount of radioactivity (about 5000 cpm) per lane on a pre-electrophoresed 16% denaturing polyacrylamide gel (*see Note 3*). Electrophorese in 1X TBE buffer at 45-W constant power until the bromophenol dye front is near the bottom of the gel. Remove the gel plates, pry apart, and transfer the gel to a bath containing 15% methanol and 10% acetic acid. Leave for 20 min, and then with the gel still attached to the glass plate, place a similar sized piece of Whatman 3MM paper on top of the gel. Carefully peel the 3MM paper with the gel attached to it. Cover the gel with plastic wrap (Saran Wrap), and dry under vacuum at 80°C for 45 min. Expose the dried gel to X-ray film at –70°C for 12–16 h with an intensifying screen. Alternatively, phosphorimager cassettes can be used for the same length of time, but at room temperature.

4. Notes

1. Purified *E. coli* uracil–DNA glycosylase can be purchased from a number of different commercial sources. On occasion, we have found that some of these preparations contain DNase activity. We therefore used PCR technology to clone the *E. coli* enzyme and place it into pGEX4T-1 (Pharmacia). The uracil-DNA glycosylase is overexpressed in *E. coli* and purified as a glutathione S-transferase fusion using affinity chromatography. Such preparations are highly stable and lack nonspecific nuclease activity. One unit of uracil-DNA-glycosylase activity is defined as the amount of enzyme that catalyzes the release of 60 pmol of uracil/min under standard reaction conditions.
2. Ordinarily, an A in the complementary strand would be positioned opposite U. However, we have used a C opposite U with complete success. Whether other mismatches are substrates for *E. coli* uracil-DNA glycosylases has not been tested by us.
3. We have used gels containing 20% polyacrylamide, but to maximize the separation of a β - and δ -elimination product, 16% gels are preferred.

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Base Excision Repair Assay Using *Xenopus laevis* Oocyte Extracts

Yoshihiro Matsumoto

1. Introduction

Base excision repair is a major mechanism for correcting modified bases. The first step of this repair mechanism is the removal of a modified base by a specific DNA-*N*-glycosylase to leave an apurinic/apyrimidinic (AP) site. Subsequently, the AP site is repaired through sequential reactions, including incision of the DNA backbone, excision of the deoxyribose phosphate (dRP), and DNA synthesis and ligation. Since we found that an extract from *Xenopus laevis* oocytes efficiently repaired AP sites (1), this repair mechanism has been further analyzed in detail. As a result, it turns out that base excision repair is carried out by two distinct pathways, the proliferating cell nuclear antigen- (PCNA) dependent pathway and the DNA polymerase β - (pol β) dependent pathway (2). Subsequent studies from several laboratories revealed that the two alternative pathways for base excision repair are observed not only in *X. laevis* oocyte extracts, but also in mammalian cell extracts (3,4; see **Chapter 24**). Thus, the in vitro system with *X. laevis* oocyte extracts can serve as a model system for base excision repair in vertebrates.

The elucidation of the detailed base excision repair mechanism was simplified by the availability of DNA substrates carrying a single lesion at a defined position. The use of these substrates enables analysis of fully repaired products as well as intermediate products at the molecular level. Two types of substrates are commonly used. One substrate is a double-stranded oligonucleotide in which one strand carries a specific lesion (e.g., see Chapter 22). The other is a circular plasmid DNA, which has been ligated in vitro with an oligonucleotide carrying a specific lesion. However, recent observations in the author's laboratory have indicated that the PCNA-dependent pathway for base excision repair

is fully functional on circular DNA but not on linear DNA, whereas the pol β -dependent pathway is able to repair efficiently both circular and linear DNAs (5). Inability of the PCNA-dependent pathway to repair AP sites on linear DNA seems to be owing to unstable loading of PCNA on linear DNA (6). Therefore, circular DNA substrates should be employed when PCNA-dependent repair is investigated. The oligonucleotide substrates are useful when the pol β -dependent pathway is exclusively analyzed.

The two pathways also have different specificities for either natural or synthetic AP sites. The pol β -dependent pathway can repair unmodified natural AP sites efficiently, but not a synthetic AP site analog, 3-hydroxy-2-hydroxymethyltetrahydrofuran, or modified AP sites (2,4). In contrast, the PCNA-dependent pathway is able to repair both forms of AP sites at the same rate. This difference is owing to distinct mechanisms for excision of AP sites in the two pathways. Both pathways are initiated by incision at the 5'-side of an AP site to leave 3'-OH and 5'-dRP termini. The pol β -dependent pathway releases a dRP residue from the 5'-incised AP site by β -elimination (7). Therefore, the synthetic or modified AP sites, which are not susceptible to β -elimination, cannot be excised in this pathway. On the contrary, the PCNA-dependent pathway employs flap endonuclease 1 (FEN-1) to excise AP sites by hydrolysis, which is not restricted by minor structural variations (4,8; Kim and Matsumoto, unpublished data).

This chapter describes an assay system for AP site repair on covalently closed circular DNA (cccDNA) with an *X. laevis* oocyte extract. The strategy for construction of circular DNA carrying a single lesion in which an oligonucleotide is ligated to a gapped heteroduplex DNA (Fig. 1A) is based on the procedures described by Naser et al. (9) and Stanssens et al. (10), and is routinely used in our laboratory with some modifications. The DNA substrates that are prelabeled with ^{32}P at a position several nucleotides either 5' or 3' of the AP site allow us to follow the fate of intermediate products from the beginning to the end of the repair reaction, and also provide a clear measure of the efficiency of the repair reaction. An alternative strategy for cccDNA construction in which cccDNA is formed by priming single-stranded DNA (ssDNA) with an oligonucleotide carrying a specific lesion and incubating with T4 DNA polymerase, ssDNA binding protein and T4 DNA ligase have been used in several laboratories (3,11,12; see also Chapters 24, 30, and 46), but is not described here.

2. Materials

2.1. Construction of Abasic Site-Containing cccDNA

1. Double-stranded DNA (dsDNA) of a modified pBS- vector, which carries a spacer fragment of several hundred base pairs between *Ava*I and *Eco*RI sites (see Note 1). pBS- is a phagemid vector from Stratagene (La Jolla, CA).

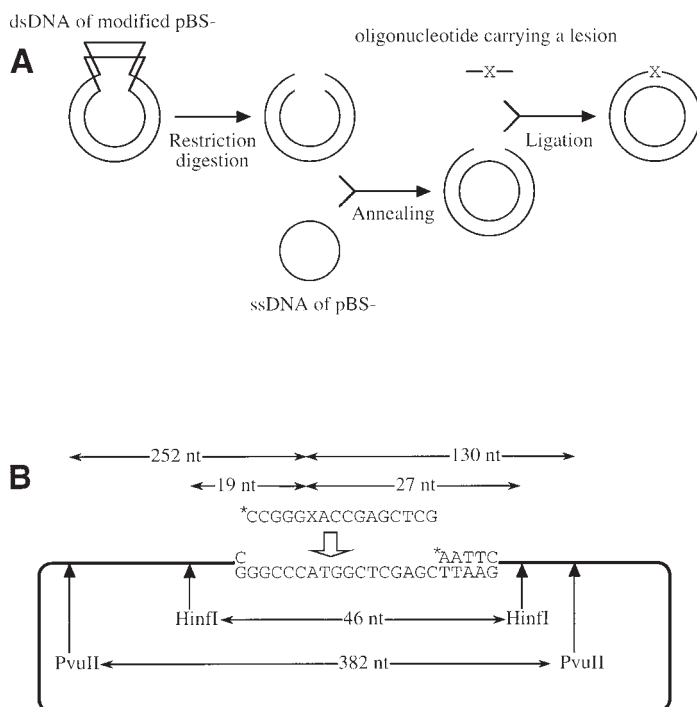


Fig. 1. Construction of cccDNA carrying a single lesion. **(A)** Strategy for cccDNA preparation. A specific lesion, either a synthetic AP site analog or a deoxyuridine, is designated as X. **(B)** Structure of the cccDNA carrying a specific lesion. The positions of 5'- and 3'-labels are indicated with an asterisks, respectively.

2. ssDNA of pBS- (without a spacer fragment): A large-scale preparation of ssDNA of pBS- can be performed by scaling up the method described by Trower (*13*) to 200 mL of culture. We use JM101 as a host strain to promote a high yield of ssDNA. The preparation thus obtained usually contains only a small quantity of ssDNA from the helper phage.
3. Oligonucleotide, 5'-CCGGGXACCGAGCTCG-3' (X is either 3-hydroxy-2-hydroxymethyltetrahydrofuran or deoxyuridine). A phosphoramidite derivative of 3-hydroxy-2-hydroxymethyltetrahydrofuran is available for automated oligonucleotide synthesis under the name of dSpacer from Glen Research Corporation, Sterling, VA.
4. Restriction enzymes: *EcoRI* (Gibco BRL, Life Technologies, Grand Island, NY), *AvaI* (Promega, Madison, WI). (See **Note 2**).
5. 10X *EcoRI* digestion buffer: 500 mM Tris-HCl, pH 8.0, 100 mM MgCl₂, 1 M NaCl (Gibco BRL).
6. 10 mg/mL Bovine serum albumin (BSA).
7. Phenol/chloroform (1:1).

8. TE buffer: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0.
9. 3 M Sodium acetate, pH 5.2.
10. Ethanol, 100%.
11. 10X Annealing buffer: 100 mM Tris-HCl, pH 7.5, 1.5 M NaCl.
12. T4 polynucleotide kinase (Gibco BRL).
13. 5X Phosphorylation buffer: 350 mM Tris-HCl, pH 7.6, 50 mM MgCl₂, 500 mM KCl, 5 mM β -mercaptoethanol (Gibco BRL).
14. 10 mM ATP.
15. T4 DNA ligase (Gibco BRL).
16. 5X Ligation buffer: 250 mM Tris-HCl, pH 7.6, 50 mM MgCl₂, 5 mM ATP, 5 mM dithiothreitol (DTT), 25% (w/v) polyethylene glycol-8000 (Gibco BRL).
17. 10 mg/mL Ethidium bromide.
18. Cesium chloride solution: 1 g cesium chloride/1 mL of TE buffer.
19. Cesium chloride-saturated isopropanol: Isopropanol is mixed 1:1 (v/v) with the cesium chloride-saturated water. The upper phase is the organic layer.
20. Centricon-30 (Amicon, Beverly, MA).
21. [γ -³²P]ATP, 4500 Ci/mmol, 10 μ Ci/ μ L; ICN, Costa Mesa, CA.
22. Calf intestinal alkaline phosphatase (CIAP) (Promega).
23. 0.25 M EDTA, pH 8.0.
24. 10 M Ammonium acetate.

2.2. Preparation of S150 Extract

1. 10 Adult female frogs, *X. laevis* (Nasco, Fort Atkinson, WI, or Xenopus I, Ann Arbor, MI).
2. DNOM buffer: 60 mM NaCl, 2 mM KCl, 1 mM sodium phosphate, pH 7.4, 5 mM HEPES-KOH, pH 7.4, 1 mM MgCl₂. Autoclave. Two liters are sufficient for preparation of S150 extract from 10 frogs.
3. Collagenase, type II (Sigma, St. Louis, MO): Make 100 mL of 0.2% (w/v) solution in DNOM buffer just before use.
4. Extraction buffer: 30 mM Tris-HCl, pH 7.9, 90 mM KCl, 2 mM EGTA, 1 mM DTT, 10 mM β -glycerophosphate. Make the buffer without DTT and β -glycerophosphate, and autoclave. Add DTT (0.5 M stock solution) and β -glycerophosphate as powder before use.

2.3. Repair Assay

1. Uracil-DNA glycosylase (Epicentre, Madison, WI or Perkin-Elmer, Foster City, CA; see Note 3).
2. 1 M HEPES-KOH, pH 7.5.
3. 1 M MgCl₂.
4. 3 M KCl.
5. 0.1 M DTT: Store in small aliquots at -20°C.
6. 0.1 M ATP: Store in small aliquots at -20°C.
7. 1 mM dNTP mixture: 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM TTP. Store in small aliquots at -20°C.

8. Extraction buffer: same as for S150 extract preparation, except that this is stored in small aliquots at -20°C .
9. 2% Sodium dodecyl sulfate (SDS).
10. Proteinase K: 1 mg/mL prepared in 50% glycerol. Store at -20°C .
11. Carrier tRNA: 0.2 mg/mL in TE buffer. Store at -20°C .
12. Stop solution: 10 mM Tris-HCl, pH 7.5, 300 mM sodium acetate, 10 mM EDTA, pH 8.0, 0.5% SDS.
13. Phenol/chloroform (1:1).
14. Ethanol, 100%.
15. *Pvu*II digestion mixture: freshly made from 10X digestion buffer (100 mM Tris-HCl, pH 7.9, 100 mM MgCl_2 , 500 mM NaCl, 10 mM DTT; New England Biolabs, Beverly, MA), *Pvu*II (Promega; 2 U/reaction; see **Note 2**), and BSA (final concentration, 0.1 mg/mL).
16. *Hinf*I digestion mixture: freshly made from 10X digestion buffer (100 mM Tris-HCl, pH 7.9, 100 mM MgCl_2 , 500 mM NaCl, 10 mM DTT; New England Biolabs), *Hinf*I (New England Biolabs; 2 U/reaction; see **Note 2**), and BSA (final concentration, 0.1 mg/mL).
17. Formamide-dye solution: 0.5% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol FF in deionized formamide, adjusted with NaOH to a neutral pH.
18. 0.1 M *bis*-Tris-HCl, pH 6.5.
19. 5 M NaBH_4 : Make fresh just before use.
20. 1 M NH_4Cl .

3. Methods

3.1. Construction of *cccDNA* Carrying a Single Lesion

3.1.1. Unlabeled *cccDNA* Carrying a Single Lesion

1. Digest 50 μg of dsDNA of the modified pBS- with 20 U of *Ava*I and 20 U of *Eco*RI in 100 μL of *Eco*RI digestion buffer (10 μL of 10X *Eco*RI digestion buffer, 1 μL of 10 mg/mL BSA; adjust the final volume with H_2O) at 37°C for 4 h.
2. Deproteinize by phenol/chloroform extraction. Back-extract with 100 μL of TE buffer, and combine the two aqueous phases.
3. Add 50 μg of ssDNA of pBS- to the linearized dsDNA.
4. Precipitate the DNA with 1/10 vol of 3 M sodium acetate and 2.5 vol of ethanol.
5. Rinse the precipitated DNA with ethanol to remove completely the salt-containing supernatant.
6. Dissolve the DNA in 200 μL of H_2O .
7. Incubate the DNA solution at 72°C for 10 min.
8. Add 22 μL of 10X annealing buffer to the DNA solution while keeping it at 72°C .
9. Cool the sample gradually to room temperature. We use a water bath with refrigerating circulation. It takes approx 1 h to reach room temperature.
10. Electrophorese a small aliquot of the sample (0.2 μg DNA) in a 1% agarose gel. Heteroduplex molecules will appear as an additional band with a slow mobility similar to that of nicked circular plasmid DNA (**Fig. 2**; see **Note 4**). If het-

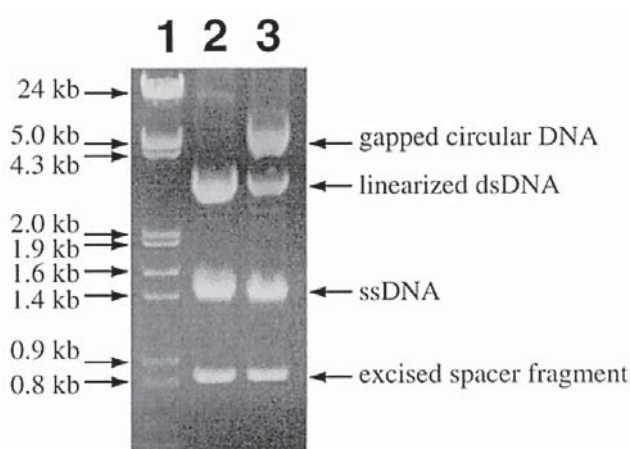


Fig. 2. Detection of heteroduplex molecules by gel electrophoresis. Small aliquots of DNA from the sample before heteroduplex formation (from **step 6** of **Subheading 3.1.1.**; lane 2) and the sample after heteroduplex formation (from **step 9** of **Subheading 3.1.1.**; lane 3) were analyzed by 1% agarose gel electrophoresis in TBE buffer with 0.5 $\mu\text{g/mL}$ ethidium bromide. Lane 1 contains λ DNA/*Eco*RI + *Hind*III markers.

eroduplex molecules have not been formed (*see Note 5*), go to **step 11** and repeat **steps 5–10**.

11. Precipitate DNA with sodium acetate and ethanol, and rinse the pellet with ethanol.
12. Dissolve DNA in 30 μL of H_2O .
13. While waiting for heteroduplex formation, phosphorylate 150 pmol of the oligonucleotide with 10 U of T4 polynucleotide kinase and nonradioactive ATP (final 0.1 mM) in 30 μL of phosphorylation buffer (6 μL of 5X phosphorylation buffer; adjust the final volume with H_2O) at 37°C for 1 h.
14. Ligate the DNA and the phosphorylated oligonucleotide with 2 U of T4 DNA ligase in 100 μL ligation buffer (20 μL of 5X ligation buffer; adjust the final volume with H_2O) at 15°C for several hours or overnight (*see Note 6*).
15. Recover the DNA by phenol/chloroform extraction and ethanol precipitation.
16. Dissolve the precipitated DNA in 200 μL of TE buffer.
17. Dissolve 3.8 g of cesium chloride in 3.5 mL of TE buffer.
18. Mix the ligated DNA, the cesium chloride solution, and 0.1 mL of 10 mg/mL ethidium bromide.
19. Transfer the DNA mixture to a tube for the VTi65 rotor (Beckman, Palo Alto, CA).
20. Fill up the tube with the cesium chloride solution, and seal the tube.
21. Centrifuge the tube at 50,000 rpm (240,000g) for more than 12 h. After centrifugation, cccDNA molecules will form a minor lower band visualized with long-wave (366 nm) UV illuminator, and linear and nicked DNA molecules form a major upper band.

22. Withdraw the cccDNA with a 3-mL syringe from the side of the tube.
23. Extract ethidium bromide from the recovered DNA sample with cesium chloride-saturated isopropanol. Repeat the extraction at least four times or until ethidium bromide is completely removed as judged with a long-wave UV illuminator.
24. Concentrate the DNA solution in a Centricon-30 by centrifugation with a JA-20 rotor (Beckman) at 5500 rpm ($\sim 3700g$) for 15–30 min until the volume decreases to <0.1 mL.
25. Add 0.5 mL of TE buffer to the DNA solution in the Centricon-30, and concentrate it in the same manner. Repeat three times to remove the cesium chloride, which otherwise tends to precipitate with ethanol.
26. Transfer the DNA solution to a 1.5-mL microcentrifuge tube.
27. Precipitate the DNA with sodium acetate and ethanol.
28. Dissolve the precipitated DNA in 100 μ L of TE buffer.
29. Estimate the concentration of the DNA. We usually digest a small aliquot of the purified DNA with *Eco*RI and compare its intensity in a 1% agarose gel with those of 10–100 ng of a standard DNA. The yield of the cccDNA is usually around 10 μ g.

Store the unlabeled cccDNA at 4°C. The uracil-containing DNA should be treated with uracil-DNA glycosylase to generate a natural AP site immediately before repair assay.

3.1.2. cccDNA Carrying a Single Lesion with a 5'-Label (see **Note 7**)

1. Follow **steps 1–12** of **Subheading 3.1.1**.
2. While waiting for heteroduplex formation, phosphorylate 60 pmol of the oligonucleotide with 5 U of T4 polynucleotide kinase and 35 μ L of [γ - ^{32} P]ATP in 45 μ L phosphorylation buffer.
3. Follow **steps 14–28** of **Subheading 3.1.1**.
4. Measure the radioactivity of the purified DNA by Cerenkov counting.
5. Dilute the DNA solution with TE buffer to provide a final concentration of 20,000 cpm/ μ L.
6. Dispense the DNA in small aliquots, and store in a radiation-shield box at 4°C.

The total radioactivity of the purified DNA is usually around 7×10^6 cpm by Cerenkov counting.

3.1.3. cccDNA Carrying a Single Lesion with a 3'-Label (see **Note 7**)

1. Digest 50 μ g of the modified pBS- dsDNA with 20 U of *Eco*RI.
2. Recover the digested DNA through phenol/chloroform extraction and ethanol precipitation.
3. Incubate the DNA with 10 U of CIAP in 100 μ L of *Eco*RI digestion buffer at 50°C for 1 h.
4. Add 10 μ L of 0.25 M EDTA to the reaction, and incubate at 75°C for 10 min.

5. Recover the CIAP-treated DNA by phenol/chloroform extraction and ethanol precipitation.
6. Phosphorylate the CIAP-treated DNA with 5 U of T4 polynucleotide kinase and 30 μL [γ - ^{32}P]ATP in 40 μL of phosphorylation buffer (*see* **Note 8**).
7. Recover the ^{32}P -labeled DNA by phenol/chloroform extraction and ethanol precipitation (with 1/4 vol of 10 *M* ammonium acetate, instead of sodium acetate). Rinse the precipitate with ethanol.
8. Digest the ^{32}P -labeled DNA with 20 U of *Ava*I in 100 μL of *Eco*RI digestion buffer at 37°C for 4 h.
9. Follow **steps 2–28** of **Subheading 3.1.1.**, and then **steps 4–6** of **Subheading 3.1.2.** The total radioactivity of the purified DNA is usually around 4×10^6 cpm by Cerenkov counting.

3.2. Preparation of S150 Extract from *X. laevis* Oocytes

The following procedures are based on the method described by Glikin et al. (**14**) with some modifications, and are designed for 10 frogs.

1. Sedate ten frogs by prolonged hypothermia in ice water. Sacrifice them by decapitation.
2. Quickly collect healthy ovaries from the frogs. Healthy ovaries have a large population of mature oocytes, each of which appears distinctly bisected. Usually around 10 g of ovary are obtained per frog. Unhealthy ovaries, which are rather gray or contain only a minor population of mature oocytes, should be discarded.
3. Wash the ovaries with DNOM buffer three times.
4. Incubate the ovaries in 100 mL of DNOM buffer with 0.2% (w/v) collagenase with gentle agitation at room temperature for 2–4 h until individual oocytes are dispersed.
5. Allow the cells to settle. Decant the buffer along with follicle cells and small immature oocytes. Wash the sediment with precooled DNOM buffer and decant. Repeat 10 times. The total volume of oocytes will be reduced to two-thirds to one-half of the initial volume.
6. Wash the oocytes with precooled extraction buffer and decant.
7. Transfer the oocytes into tubes for an SW41 rotor (Beckman), and remove excess buffer surrounding the oocytes (**Fig. 3A**).
8. Centrifuge at 35,000 rpm (150,000*g*, avg.) for 30 min at 4°C.
9. Withdraw the light turbid layer (=S150; **Fig. 3B**) from the side of the tube with a syringe.
10. Quickly freeze the S150 extract in small aliquots (~50 μL), and store at –80°C. The S150 extract can be stored under this condition for at least 2 yr without losing AP site repair activity.

The protein concentration and yield of S150 are usually around 10 mg/mL and 1.5 mL/frog, respectively.

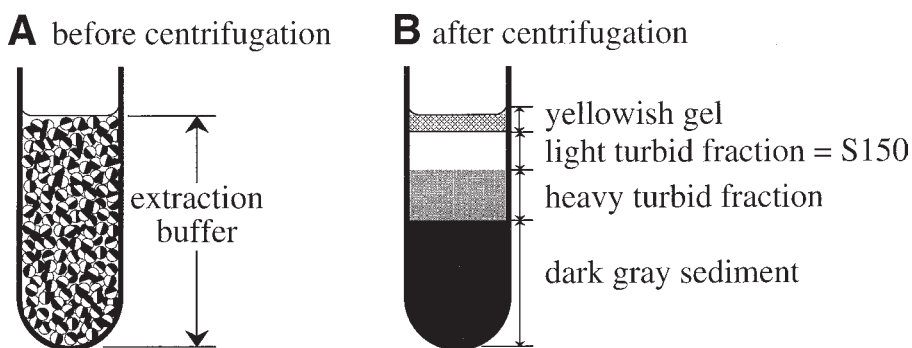


Fig. 3. Schematic diagram of the preparation of S150 extract.

3.3. AP Site Repair Assay

3.3.1. Synthetic AP Site Repair

1. Prepare a mixture of unlabeled cccDNA (10 ng/reaction) and either 5'- or 3'-prelabeled cccDNA (200–1000 cpm per reaction), both carrying a synthetic AP site, sufficient for one experiment consisting of multiple reactions. The volume should be <2 μ L/reaction.
2. Mix the following reagents (the volumes are provided as per reaction and should be scaled up for experiments involving multiple samples): 0.4 μ L of 1 M HEPES-KOH, pH 7.5, 0.2 μ L of 1 M $MgCl_2$, 0.5 μ L of 3 M KCl, 0.2 μ L of 0.1 M DTT, 1 μ L of 0.1 M ATP (see **Note 9**), 0.4 μ L of 1 mM dNTP mixture, the cccDNA mixture, and H_2O to adjust the volume per reaction to 15 μ L.
3. Dispense 15 μ L each of the combined mixture to 1.5-mL microcentrifuge tubes, and preincubate at 25°C.
4. Dilute the S150 extract with extraction buffer to provide an appropriate protein concentration (usually 0.4–5.0 mg/mL).
5. Start the reaction by adding 5 μ L of the diluted S150 extract to each reaction tube.
6. Incubate the reaction mixture at 25°C for an appropriate time (usually 5–60 min).
7. Stop the reaction by the addition of 6 μ L of 2% SDS to each tube.
8. Add 2 μ L of 1 mg/mL proteinase K and 2 μ L of 0.2 mg/mL carrier tRNA to each tube, and incubate at 37°C for 30 min.
9. Add 150 μ L of stop solution.
10. Recover the DNA by phenol/chloroform extraction and ethanol precipitation. Remove the residual supernatant completely.
11. Dissolve the precipitated DNA in 7 μ L of either *Pvu*II digestion mixture or *Hinf*I digestion mixture (see **Note 10**, **Fig. 1B**).
12. Incubate the digestion mixtures at 37°C for 1 h.
13. Add 7 μ L of formamide-dye mixture to each tube.
14. Boil the mixtures for 1 min, and place on ice water.

15. Load the chilled mixtures on either 8 *M* urea-containing 6% polyacrylamide gel (7 cm high) for *Pvu*II-digested samples or 8 *M* urea-containing 20% polyacrylamide gel (20 cm high) for *Hinf*I-digested samples.
16. Electrophorese the samples in the 6% gel at 200 V for 50 min or in the 20% gel at 1200 V for 1.5 h.
17. Dry the 6% gel on a 3MM paper, or transfer the 20% gel onto a used X-ray film.
18. Visualize the radioactive bands by autoradiography with X-ray film or using a phosphorimager.

3.3.2. Natural AP Site Repair

Natural AP sites are not stable during storage. Therefore, the deoxyuridine site should be converted to an AP site by uracil-DNA glycosylase just before use, and the residual AP sites should be reduced to a chemically stable form immediately after the repair reaction. Complete removal of uracil is essential for clear interpretation of results.

1. Prepare a mixture of the uracil-containing DNA as described in **step 1** for synthetic AP site repair (**Subheading 3.3.1.**)
2. Incubate the mixed uracil-containing DNA with uracil-DNA glycosylase (0.1 U/reaction) at 37°C for 1 h.
3. Follow **steps 2–7** of **Subheading 3.3.1.**
4. Add 70 μ L of 0.1 *M* bis-Tris-HCl (pH 6.5) to each tube. This stabilizes the natural AP site, which is labile at alkaline pH.
5. Make a fresh solution of 5 *M* NaBH₄. Add 7 μ L of 5 *M* NaBH₄ to each tube. Leave for 10 min at room temperature with the cap open to avoid explosion.
6. Add 2 μ L of 1 mg/mL proteinase K to each tube, and incubate at 37°C for 30 min.
7. Add 100 μ L of 1 *M* NH₄Cl and 2 μ L of 0.2 mg/mL tRNA to each tube.
8. Follow **steps 10–18** of **Subheading 3.3.1.**

4. Notes

1. Insertion of a spacer fragment into a phagemid vector is optional. However, this modification is helpful in checking for complete digestion by restriction enzymes and for ligation efficiency (the small spacer fragment should disappear after ligation).
2. Restriction enzymes from commercial sources are sometimes contaminated with AP endonuclease and/or exonuclease. Try to use enzymes of the best quality, avoid overdigestion, and perform controls to rule out contamination.
3. Make sure that uracil-DNA glycosylase is not contaminated with AP lyase.
4. The relative mobility of the gapped circular DNA to the linear DNA depends on electrophoretic field strength. The electrophoresis shown in **Fig. 2** was performed at 3.3 V/cm.
5. Most cases when heteroduplex molecules are not formed result from residual salt after ethanol precipitation which may interfere with denaturation of dsDNA.

6. In the case with a nonradioactive oligonucleotide, this procedure employs a severalfold excess of the oligonucleotide to permit efficient use of the heteroduplex DNA.
7. When ^{32}P -labeling is performed, appropriate precautions should be used to avoid contamination.
8. Fifty micrograms of pBS- dsDNA represents approx 50 pmol (a little less with the modified pBS-) of *Eco*RI termini available for labeling. An excess amount of ATP is needed for efficient phosphorylation of DNA ends.
9. The repair activity of some S150 extracts is very sensitive to the ATP concentration, and its optimal condition is variable between batches of the extracts. Therefore, the author recommends determining the optimal ATP concentration in a preliminary experiment.
10. Since AP endonuclease in the S150 extract from *X. laevis* oocytes is abundant, all the AP sites in the assay are usually incised. However, most mammalian cell extracts tested in the author's laboratory contained a relatively low activity of AP endonuclease, and therefore leave some AP sites uncleaved, making the unrepaired DNA indistinguishable from the repaired products by size. In this case, AP endonuclease should be added to the restriction digestion mixture. *E. coli* endonuclease IV is commercially available from Trevigen.

Acknowledgments

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In Vitro Base Excision Repair Assay Using Mammalian Cell Extracts

**Guido Frosina, Enrico Cappelli, Paola Fortini,
and Eugenia Dogliotti**

1. Introduction

Base excision repair (BER) is a major cellular repair mechanism that corrects a broad range of DNA lesions (for a review, *see 1*). BER deals with DNA damage generated not only by environmental genotoxins, like ionizing radiation, alkylating agents and oxidative reagents, but also by endogeneously produced oxygen radicals and other reactive species. Therefore, its correct functioning is very important for genome stability and cell viability (2,3). The primary pathway for BER involves the recognition by a DNA glycosylase of the damaged base followed by cleavage of the *N*-glycosyl bond to generate an apurinic/apyrimidinic (AP) site. The AP site is then recognized by an endonuclease. The major AP endonucleases cleave hydrolytically the phosphodiester bond on the 5'-side of the AP site. A phosphodiesterase then excises the generated 5'-deoxyribose phosphate terminus to leave a single nucleotide gap. This gap can then be filled by a DNA polymerase (polymerase β in mammalian cells) and the nick sealed by a DNA ligase. In eukaryotes, an alternative BER pathway that involves the replacement of more than a single residue is also present (4–6). Repair synthesis, which is dependent upon proliferating cell nuclear antigen (PCNA), occurs on the 3'-side of the damaged residue and involves the replacement of two to six nucleotides. It is likely that these two repair mechanisms have evolved to repair structurally distinct lesions.

The knowledge of human nucleotide excision repair has been greatly improved by the development and use of a cell-free in vitro repair assay (7; *see* Chapter 29). This same methodology has been successfully applied in the past few years to the analysis of the BER process (4–6,8,9). This chapter details a

BER synthesis assay using mammalian cell extracts and, as DNA substrate, a plasmid containing a single AP site. AP sites are typical BER lesions, since they are generated as intermediates during the repair process itself. For many research questions, randomly depurinated plasmid DNA, which is easier to produce, can be used as an alternative repair substrate. However, only DNA containing a single lesion at a defined site is amenable to fine mapping of the repair patches, thus allowing discrimination between the two BER pathways.

2. Materials

2.1. Construction of DNA Substrates Containing a Single AP Site (see Note 1)

1. Single-stranded circular DNA. Any plasmid (*see Note 2*) which contains the origin of replication of single-stranded phage DNA is suitable. We currently use the phagemid pGEM-3Zf (+) (Promega) (**Fig. 1**) to produce single-stranded (+) pGEM-3Zf DNA. Materials listed below (**items 2–4**) are required to prepare single-stranded circular DNA from this plasmid. A method for preparing single-stranded DNA is described in Chapter 46.
2. M13K07 helper phage DNA (Promega).
3. Kanamycin stock solution (10 mg/mL) dissolved in distilled water. Sterilize by filtration, and store in aliquots at -20°C .
4. 2x YT bacterial growth medium: 10 g yeast extract, 16 g Bacto-tryptone, and 5 g NaCl/L, dissolved in water and autoclaved.
5. Primers for in vitro DNA replication:
 - a. An oligonucleotide which contains a single uracil residue (e.g., 5'GATCCTCTAGAGUCGACCTGCA3') and
 - b. A control oligonucleotide (e.g., 5'GATCCTCTAGAGTCGACCTGCA3') (**Fig. 1**). These oligonucleotides are prepared by automated DNA synthesis.
6. T4 Polynucleotide-DNA-kinase (PNK) (New England Biolabs).
7. Stock solutions for kinasing of primers:
 - a. 1 M Tris-HCl, pH 7.6;
 - b. 0.2 M MgCl_2 ;
 - c. 1 M dithiothreitol (DTT).Autoclave solutions a and b, and store at -20°C . Filter-sterilize solution c, and store at -20°C .
8. TE (pH 8.0): 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. Sterilize by autoclaving.
9. Sephadex G-50 (medium) dissolved in TE, pH 8.0 (*see Note 3*). Store at 4°C in a screw-capped bottle.
10. 10X Annealing buffer: 200 mM Tris-HCl, pH 7.4, 20 mM MgCl_2 , 0.5 M NaCl. Sterilize by filtration, and store in aliquots at -20°C .
11. 10X in vitro synthesis buffer: 175 mM Tris-HCl, pH 7.4, 37.5 mM MgCl_2 , 215 mM DTT, 7.5 mM ATP, and 0.4 mM each dNTP. Store in aliquots at -20°C (*see Note 4*).
12. 0.1 M ATP stock solution. ATP is dissolved in distilled water. Sterilize by filtration, and store in aliquots at -80°C .

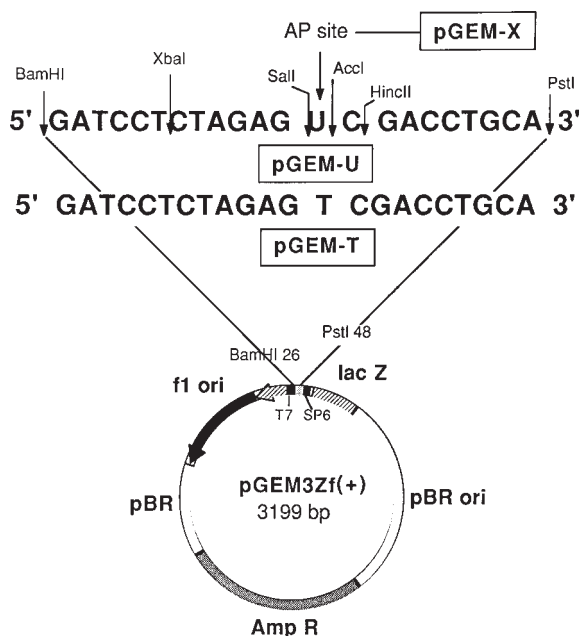


Fig. 1. Scheme of the circular duplex DNA molecules used as substrates. pGEM-T and pGEM-U plasmids were obtained by priming single-stranded (+) pGEM-3Zf DNA with the indicated oligonucleotides and performing in vitro DNA synthesis as described. The single AP site-containing plasmid, pGEM-X, was obtained by incubation of pGEM-U with uracil-DNA-glycosylase. The positions of the AP site and the restriction sites in its proximity, which may be utilized for fine mapping of the repair patch, are also indicated. Reprinted with permission from ref. 5.

13. T4 gene 32 protein (single-strand DNA binding protein); T4 DNA polymerase holoenzyme; T4 DNA ligase (Boehringer Mannheim).
14. *Escherichia coli* uracil-DNA-glycosylase (Ung protein) (see **Note 5**).
15. 10X *E. coli* uracil-DNA-glycosylase buffer: 70 mM HEPES-KOH, pH 7.8, 50 mM β -mercaptoethanol, 20 mM Na_2EDTA , 350 mM NaCl. Sterilize by filtration, and store in aliquots at -20°C .
16. Solid cesium chloride.
17. Ethidium bromide solution (10 mg/mL): Ethidium bromide is dissolved in distilled water. Store in aliquots at 4°C in dark bottles. Ethidium bromide is a potent mutagen. Wear gloves and a mask when weighing it out.
18. 1-Butanol saturated with water.
19. 7 M Ammonium acetate: Dispense into aliquots and sterilize by autoclaving.
20. 3 M Sodium acetate, pH 5.2: Dispense into aliquots and sterilize by autoclaving.
21. Phenol/chloroform/isoamyl alcohol (25:24:1) saturated with TE (pH 8.0).
22. Chloroform/isoamyl alcohol (24:1).

23. Ethanol, 100%.
24. 1% Agarose gel.

2.2. Preparation of Whole-Cell Extracts

1. 0.25% Trypsin/Phosphate-buffered saline (PBS) (Gibco BRL).
2. Hypotonic lysis buffer (pH 7.9): 10 mM Tris-HCl, 1 mM EDTA, 5 mM DTT, 0.5 mM spermidine, 0.1 mM spermine. Store at 4°C.
3. Sucrose-glycerol buffer (pH 7.9): 50 mM Tris-HCl, 10 mM MgCl₂, 2 mM DTT, 25% sucrose (molecular biology grade), 50% glycerol (Fluka). Store at 4°C.
4. Dialysis buffer (pH 7.9): 25 mM HEPES-KOH, 100 mM KCl, 12 mM MgCl₂, 1 mM EDTA, 17% glycerol, 2 mM DTT (add just before use), pH to 7.9, with 5 M KOH. Store at 4°C.
5. Protease inhibitors (all from Sigma):
 - a. 500 mM (87 mg/mL) phenylmethylsulfonyl fluoride (PMSF) dissolved in acetone. Store at -20°C in dark glass bottle;
 - b. 5 mg/mL pepstatin A, chymostatin, antipain and aprotinin, each dissolved in sterile 20% dimethylsulfoxide. Store at -20°C;
 - c. 5 mg/mL leupeptin dissolved in sterile water. Store at -20°C. Protease inhibitors are highly toxic: wear gloves and handle with care.
6. Solid ammonium sulfate.
7. Saturated ammonium sulfate solution neutralized to pH 7.0 with NaOH.
8. Polyallomer tubes (Beckman) for a SW41 or SW55 rotor (or equivalent).

2.3. In Vitro Repair Assay

1. 5X reaction buffer: 25 mM MgCl₂, 200 mM HEPES-KOH, pH 7.8, 2.5 mM DTT, 10 mM ATP, 100 μM dGTP, 100 μM dCTP, 100 μM TTP, 100 μM dATP, 200 mM phosphocreatine, 1.8 mg/mL bovine serum albumin (BSA). Store in aliquots at -80°C for up to 2 mo.
2. Creatine phosphokinase (CPK) (type I, Sigma), 2.5 mg/mL dissolved in 5 mM glycine, pH 9.0, 50% glycerol. Store in aliquots at -20°C.
3. TE (pH 7.8): 10 mM Tris-HCl, pH 7.8, 1 mM EDTA. Sterilize by autoclaving.
4. pGEM-X and pGEM-T plasmid substrates (**Fig. 1**) dissolved in TE, pH 7.8 at 100 ng/μL. Store in aliquots at -80°C.
5. α-³²P-labeled deoxynucleotides (α-³²P TTP or α-³²P dCTP when pGEM-X is used as substrate), 3000 Ci/mmol, 10 mCi/mL, aqueous solution. Use a recently labeled deoxynucleotide to maximize the sensitivity of the repair assay. Perform all the steps behind a shield for ³²P and wear protective clothing (gloves and glasses) for β-emitting radioisotopes.
6. 3 M KCl, sterilize and store at 4°C.
7. Appropriate α-³²P-5' end-labeled DNA size markers (store at -20°C).
8. Whole-cell extracts (store at -80°C or in liquid nitrogen).
9. RNase A: Dissolve at a concentration of 2 mg/mL in 10 mM Tris-HCl, pH 7.5, and 15 mM NaCl. Heat for 15 min at 100°C and allow to cool slowly to room temperature. Store in aliquots at -20°C.

10. Proteinase K: Dissolve at a concentration of 2 mg/mL in H₂O. Store in aliquots at -20°C.
11. Restriction enzymes with suitable 10X reaction buffers.
12. Denaturing loading buffer: 80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue.
13. Polyacrylamide gel electrophoresis equipment.
14. PhosphorImager or liquid scintillation counter.

3. Methods

3.1. Construction of DNA Substrates

1. Phosphorylate both the control and the uracil-containing oligonucleotides by using PNK according to standard procedure (*see Note 6*). Remove the free ATP by chromatography on, or centrifugation through, small columns of Sephadex G-50.
2. Anneal the phosphorylated oligonucleotide to single-stranded (ss) pGEM-3Zf(+) DNA by performing the following program in a thermal cycler: 75°C for 5 min; from 75°C to 30°C over 30 min. Assemble each annealing reaction in a tube appropriate for the thermal cycler (usually a 0.5-mL microcentrifuge tube), as follows (*see Note 7*):

Phosphorylated primer (40 ng/μL)	10.5 μL
ss pGEM-3Zf(+) (200 ng/μL)	10 μL
10X annealing buffer	3 μL
Distilled water	6.5 μL

3. Spin down the samples and assemble the *in vitro* replication reactions by adding to each tube:

10X synthesis buffer	3.6 μL
T4 gene 32 protein (5 mg/mL)	0.5 μL
T4DNA ligase (1 U/μL)	1 μL
T4 DNA polymerase (1 U/μL)	1 μL
4. Incubate for 5 min on ice, then for 5 min at room temperature, and finally for 90 min at 37°C.
5. Transfer and pool the replication reactions in a 1.5-mL microcentrifuge tube. Purify the DNA by standard procedure. Briefly, extract the DNA with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) saturated with TE, followed by extraction with an equal volume of chloroform/isoamyl alcohol (24:1). Precipitate the DNA by adding 1/4 vol of 7 M ammonium acetate and 2 vol of cold pure ethanol. Keep the samples at -80°C overnight.
6. Centrifuge for 30 min at top speed (approx 15,000g) in an Eppendorf centrifuge at 4°C, and wash the pellet with 70% ethanol. Dry the pellet using a Speed Vac for 2-3 min, and resuspend it in 30 μL of TE (pH 8.0).
7. Analyze the replication products on a 1% agarose gel (*see Note 8*).
8. Purify the closed circular duplex DNA molecules by equilibrium density gradient centrifugation with cesium chloride (*see Note 9*).

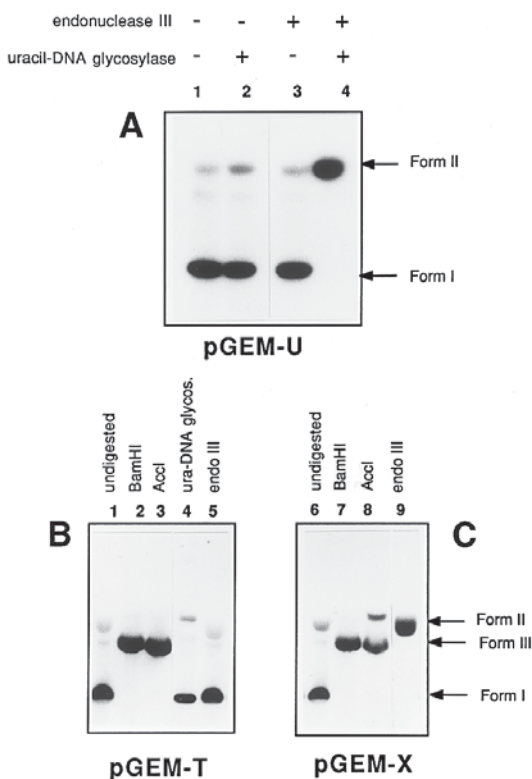


Fig. 2. Characterization of the single AP site-containing substrate. (A) Lane 1: construct containing a single uracil residue, pGEM-U; lane 2: after incubation with uracil-DNA-glycosylase; lane 3: after incubation with endonuclease III; lane 4: after incubation with uracil-DNA-glycosylase followed by incubation with endonuclease III. (B) Lane 1: construct containing the control oligonucleotide, pGEM-T; lane 2: after digestion with *Bam*HI (20 U); lane 3: after digestion with *Acc*I (20 U); lane 4: after incubation with uracil-DNA-glycosylase; lane 5: after incubation with endonuclease III. (C) Lane 6: construct containing a single AP site, pGEM-X; lane 7: after digestion with *Bam*HI (20 U); lane 8: after digestion with *Acc*I (20 U); lane 9: after incubation with endonuclease III. Reprinted with permission from ref. 5.

9. Incubate the plasmid molecules containing a single uracil residue (pGEM-U, **Fig. 2A**) with *E. coli* uracil-DNA glycosylase to create circular duplex DNA containing a single AP site (pGEM-X; **Fig. 2C**; see **Note 10**).

3.2. Preparation of Whole-Cell Extracts (see **Notes 11–14**)

1. Grow cells (see **Note 15**) in monolayers consisting of up to $0.6\text{--}1.2 \times 10^9$ cells in 850 cm² roller bottles. Harvest the cells when they are just confluent. In the case

of CHO cells (*see* **Note 16**), one roller bottle yields approx 1×10^8 cells. Alternatively, cells growing in suspension, like lymphoblastoid cell lines, may be used. Harvest cells from 1–2 L of an exponentially growing culture when the cell density reaches $6\text{--}8 \times 10^5/\text{mL}$.

2. Wash the monolayers twice with sterile PBS. Detach the cells by incubation with 0.25% trypsin/PBS. Add complete growth medium and pellet the cells by 15 min of centrifugation at 1300 rpm ($\sim 280g$). Resuspend the cells in 150 mL cold (4°C) PBS and pellet again.
3. Resuspend the cells in PBS, and count them. Spin at 1300 rpm ($\sim 280g$) for 15 min. Carefully remove the supernatant. Measure the packed cell volume (PCV). This is usually about 1 mL for $\sim 0.6 \times 10^9$ cells. At this step, the pellet can be frozen on dry ice and stored at -80°C .
4. Resuspend the cells in 4 PCV of hypotonic lysis buffer containing the following protease inhibitors/1 mL of PCV: 5 μL of 87 mg/mL PMSF; 0.5 μL each of 5 mg/mL leupeptin, pepstatin, chymostatin, antipain, and aprotinin. Leave the cells to swell for 20 min on wet ice. If the pellet had been frozen, add first the hypotonic lysis buffer containing the protease inhibitors and, keeping the tube on ice, resuspend the cell pellet.
5. Pour the cell suspension in a 20-mL glass homogenizer with a Teflon pestle and homogenize on ice with 20 strokes.
6. All of the following steps are performed in a cold room (4°C). Pour the homogenate into a glass beaker (50 mL for 1 mL PCV) on ice over a magnetic stirrer. Stir very slowly (about 60 rpm).
7. Add dropwise 4 PCV of sucrose/glycerol buffer. Mix it well.
8. Add dropwise 1 PCV of saturated ammonium sulfate. The viscosity of the solution gradually increases. Stirring must be as slow as possible to avoid fragmentation of high-mol-wt cellular DNA that should sediment in the next ultracentrifugation step. Stir for 30 min.
9. Pour (do not pipet) the viscous solution into polyallomer tubes. The tubes must be filled to the top. Otherwise, they will collapse in the next ultracentrifugation step.
10. Centrifuge for 3 h at 37,000 rpm with a SW41 rotor (235,000g) or at 42,000 rpm with a SW55 rotor (214,000g). For larger extract volumes, use a Ti60 rotor at 41,000 rpm (226,000g) and thick-wall polycarbonate tubes. Set the temperature at 2°C .
11. Carefully remove the tubes from the centrifuge. With a Pasteur pipet, discard aggregates that lie on the meniscus. With another Pasteur pipet, remove the supernatant leaving the last 1 mL above the pellet in the tube. (The latter fraction contains high-mol-wt DNA that has not fully pelleted.) Measure the volume of supernatant (usually 6–7 mL for 1 mL PCV).
12. Transfer the supernatant to a 30-mL Corex tube (or polyallomer tube for a SW28 rotor) containing a magnetic stir bar. Stir on ice at normal speed (300–600 rpm). Slowly add 0.33 g of pure solid ammonium sulfate/mL of supernatant. When it is dissolved, add 10 μL of 1 M NaOH/g of ammonium sulfate added. Continue stirring for 30 min.

13. Remove the magnetic stirring bar. Centrifuge for 20 min at 8000 rpm (10,500g) in an HB-4 rotor (when using Corex tubes) or in SW28 rotor (11,500g) (when using polyallomer tubes) at 2°C.
14. Remove the supernatant with a Pasteur pipet, leaving the pellet as dry as possible.
15. Resuspend the pellet in a small amount of dialysis buffer (0.05 vol of the high-speed supernatant). Do not try to dissolve the pellet, just collect the suspension (which should be thick and milky) by scraping with the end of a 1-mL Gilson tip, but not pipeting up and down.
16. Dialyze for 1.5–2 h in 500 mL of extract dialysis buffer. Change the buffer and dialyze for another 10 h. Do not dialyze longer, since significant amounts of precipitate will form.
17. Remove the dialysate and centrifuge in Eppendorf tubes. Spin for 10 min at 14,000 rpm (15,800g) to remove any precipitate. Transfer the supernatant to a fresh tube on ice.
18. Dispense 50- μ L aliquots into conical cryotubes. Freeze immediately on dry ice, and store in liquid nitrogen or at –80°C. In the latter case, a progressive decrease in repair activity will be observed starting 6 mo after preparation.
19. For an active extract, recover about 1 mL of extract/1 mL of PCV. The protein concentration should be 10–15 mg/mL.

3.3. In Vitro Repair Assay (see Notes 17–19)

3.3.1. Repair reaction

1. Premix appropriate amounts of 5X reaction buffer and CPK in order to run two more reactions than needed. Each 50- μ L reaction requires 10 μ L of 5X buffer and 1 μ L of CPK. Keep these on ice.
2. Add to the mix 3 M KCl to obtain a total final salt concentration of 70 mM KCl. Remember to take into account the contribution of KCl given by the extracts (dissolved in dialysis buffer containing 100 mM KCl) and by the other buffers used.
3. Assemble on dry ice the DNA substrates (pGEM-X and pGEM-T, kept at –80°C) and cell extracts (kept at –80°C or in liquid nitrogen), and keep frozen on dry ice.
4. Mix the appropriate amounts of labeled deoxynucleotide (α -³²P-TTP or α -³²P-dCTP in the case of pGEM-X) (see **Notes 20** and **21**) and H₂O. Each reaction includes 0.2 μ L (2 μ Ci) of labeled deoxynucleotide and 4.8 μ L of H₂O. Make a mix for two more reactions than needed.
5. Add the components to microcentrifuge tubes on wet ice in the following order (**Table 1**; see **Note 22**):
 - a. Extract buffer (the amount depends on the amount of extract added, up to a total volume of 20 μ L) to the bottom of the tubes.
 - b. TE (pH 7.8) (up to a total volume of 50 μ L).
 - c. Plasmid DNA to the bottom of the tubes.
 - d. 11 μ L of 5X reaction buffer/CPK/KCl to the side of the tubes.
 - e. 5 μ L of α -³²P-dNTP/H₂O to the side of the tubes.
6. Quick-thaw the cell extract, add the appropriate amounts, and vortex.

Table 1
Basic Composition of a Single Repair Reaction

Component	μL
5X reaction buffer	10
CPK (2.5 mg/mL)	1
α- ³² P-dNTP (10 μCi/μL)	0.2
H ₂ O	4.8
pGEM-X or pGEM-T (100 ng/μL)	3
Cell extract	Variable ^a
Dialysis buffer	Variable ^b
TE (pH 7.8)	Up to 50

^aUsually 20–200 μg protein are used.

^bThe total amount of dialysis buffer should be the same in all reactions.

7. Briefly spin the tubes in a microcentrifuge.
8. Incubate at 30°C for 1 h (*see Note 23*). Stop the reactions by adding EDTA to a final concentration of 20 mM on wet ice (*see Note 24*).

3.3.2. DNA purification

1. Briefly spin the tubes in a microcentrifuge.
2. Add 2 μL of 2 mg/mL RNase A (80 μg/mL final concentration). Incubate at 37°C for 10 min.
3. Add 3 μL of 10% SDS (0.5% final concentration) and 6 μL of 2 mg/mL proteinase K (190 μg/mL final concentration). Incubate at 37°C for 30 min.
4. Extract and precipitate the DNA as in **step 5 of Subheading 3.1**.
5. Pellet the DNA by centrifugation in a microcentrifuge at 14,000 rpm (15,800g) at 4°C.
6. Gently remove the ethanol with a Gilson micropipet keeping the tip as far as possible from the DNA pellet. Leave 100–200 μL above the pellet.
7. Wash the pellet by adding 1 mL of cold (–20°C) 70% ethanol.
8. Centrifuge at 14,000 rpm (15,800g) for 30 min at 4°C.
9. Carefully remove the ethanol without disturbing the DNA pellet.
10. Dry the DNA pellet at room temperature. Do not overdry the pellet because it will become difficult to redissolve.
11. Incubate the DNA with the appropriate restriction endonuclease according to the manufacturer's instructions (*see Note 25*).
12. Extract and precipitate the DNA as in step 5 of **Subheading 3.1**.
13. Resuspend the precipitate in 4 μL of Tris-EDTA. Vortex and then add 10 μL of denaturing loading buffer to the tubes. Vortex the DNA.
14. Heat the samples at 95°C for 2 min.
15. Load onto a 15% polyacrylamide gel containing 7 M urea in 90 mM Tris-borate/2 mM EDTA (pH 8.8).

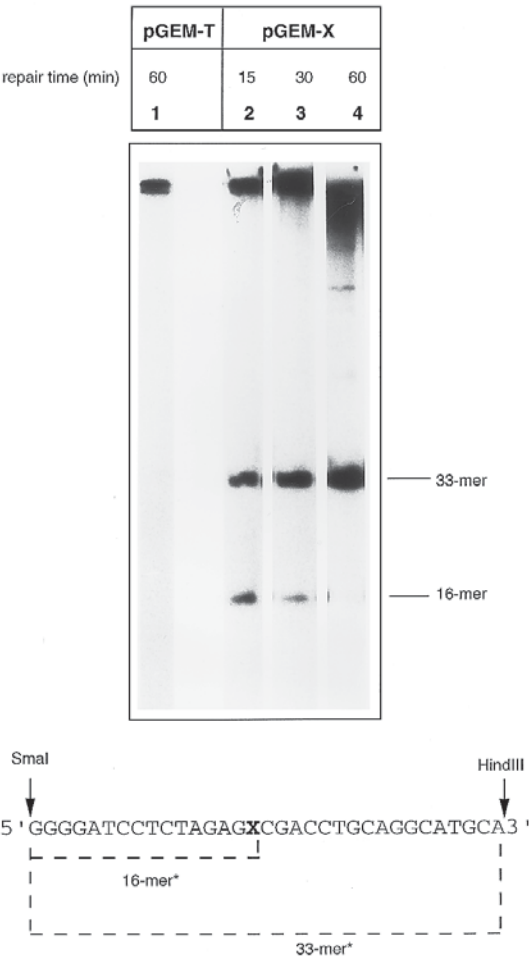


Fig. 3. Kinetics of repair replication and ligation of a single abasic site by CHO-9 whole-cell extracts. Top, autoradiograph of a denaturing polyacrylamide gel. Repair replication was performed in the presence of α - ^{32} P-TTP. pGEM-T (lane 1) or pGEM-X (lanes 2–4) were digested with *Sma*I and *Hind*III to release the 33-mer, which originally contained the AP site. pGEM-X was incubated with cell extracts for 15 (lane 2), 30 (lane 3), and 60 (lane 4) min. Unligated repair products arising from the single nucleotide insertion pathway (16-mer) are visible at the bottom of the gel after 15 (lane 2) and 30 (lane 3) min of repair time. Bottom, scheme of the expected repair products.

- 16. Electrophorese the samples at 30 mA for 60–90 min at room temperature.
- 17. Fix the gel in 10% methanol, and 10% acetic acid for 20 min.
- 18. Dry the gel and expose it to X-ray film in a cassette with intensifying screens. Store the gel at -80°C . The results of such an analysis are shown in **Fig. 3**.

19. Quantitate the repair incorporation by phosphorimager analysis or electronic autoradiography. Alternatively, excise the band from the gel and count it in a liquid scintillation counter.

4. Notes

1. DNA substrates containing multiple AP sites can also be used (8). This type of DNA substrate is much easier to produce, although it does not allow discrimination between the two BER pathways. Briefly, depurinated plasmid DNA can be obtained as follows:
 - a. Incubate plasmid DNA in 1X depurination buffer (10 mM sodium citrate, 100 mM sodium chloride, pH 5.0) at 70°C for various times (e.g., 15, 30, 45, and 60 min).
 - b. Precipitate the DNA by standard procedure.
 - c. Isolate pure supercoiled plasmid forms by equilibrium density gradient centrifugation with cesium chloride (*see Note 9*).
 - d. Measure the number of AP sites by digestion of the plasmid DNA with an AP-endonuclease, like *E. coli* endonuclease III (*Nth* protein), followed by 1% agarose-gel electrophoresis and densitometric scanning of the relative amounts of supercoiled and nicked forms. The number of abasic sites is calculated by Poisson analysis.
 - e. Store the depurinated plasmid DNA in aliquots at -80°C.
2. The standard alkaline lysis method for DNA plasmid preparation should be avoided, since the alkaline lysis step may induce alkali-labile sites, which would increase the background incorporation. The method of choice for plasmid preparation involves a neutral lysis step by SDS (10).
3. Slowly add 30 g of Sephadex G-50 (medium) to 250 mL of TE (pH 8.0) and make sure the powder is well dispersed. Autoclave and allow to cool to room temperature. Decant the supernatant, and replace with an equal volume of TE, pH 8.0.
4. Sterile solutions should be used to prepare the in vitro replication buffer. One hundred millimolar sterile stock solutions of deoxyribonucleotide triphosphates can be purchased from Pharmacia.
5. *E. coli* uracil-DNA-glycosylase was kindly provided by S. Boiteux, Institute Gustave Roussy, Villejuif, France. Specific activity was 3.1×10^5 U/mg. This enzyme is also commercially available.
6. Phosphorylation of the primer.
 - a. Mix:

1 M Tris-HCl, pH 7.5	3 μ L
0.2 M MgCl ₂	1.5 μ L
1 M DTT	1.5 μ L
1 mM ATP	13 μ L
T4 PNK (20 U/ μ L)	1 μ L
22-mer 1 μ g/ μ L)	2 μ L
H ₂ O	8 μ L

- b. Incubate at 37°C for 45 min, and then at 65°C for 10 min in order to inactivate PNK.
7. In our experimental system, the optimal molar ratio of phosphorylated primer (22-mer) to single-stranded plasmid DNA (3.2 kb) is 30:1. This ratio should be established experimentally on a case-by-case basis.
8. A successful in vitro replication reaction produces almost 90% closed circular duplex molecules. Ten percent of the replication products are usually molecules containing nicks or short gaps (which run as Form II). Occasionally, aberrant replication products, which migrate faster than closed circular duplex DNA (Form I), are detected on the gel. To avoid artifacts, it is extremely important to isolate closed circular molecules from the replication products.
9. Closed circular DNA can be purified by centrifugation in cesium chloride-ethidium bromide gradients by standard procedure (10). We suggest:
 - a. To use polyallomer Quick-Seal centrifugation tubes (13 × 51 mm) for a Beckman Type-65 rotor.
 - b. To avoid loading more than 30 µg of DNA/tube.
 - c. To check carefully the final density of the cesium chloride solution (0.5 g added to 10 mL, $\eta = 1.3905$).
 - d. To centrifuge at 55,000 rpm (290,000g) for 15 h at 18°C (using a VTi 65 rotor).

Following collection of the closed circular plasmid DNA from the tube, remove the ethidium bromide by water-saturated butanol extraction, and dialyze the aqueous phase against several changes of TE, pH 8.0. Concentrate the DNA by butanol extraction to a final volume of 200–300 µL, and then precipitate the DNA by adding 1/10 vol of 3 M sodium acetate and 2 vol of pure cold ethanol. Leave the sample at –80°C for at least 30 min, and recover the DNA by centrifugation at 14,000 rpm (15,800g) for 30 min. Resuspend the pellet in an appropriate volume of TE (pH 8.0), and run the sample in a 1% agarose gel. The relative amount of nicked circular forms (Form II) should not exceed 5% of the total DNA molecules.

10. To confirm that these plasmid molecules contain a single abasic site, digest a small aliquot with an AP-endonuclease (see **Note 1**). A complete conversion from closed circular (Form I) to nicked (Form II) forms should be observed (see **Fig. 2C**). Conversely the plasmid molecules containing the control oligonucleotide (pGEM-T) should not be cleaved (**Fig. 2B**).
11. The procedure for preparation of whole-cell extracts is basically that described by Manley et al. (11,12) for transcription-competent mammalian cell extracts with minor modifications (7,13).
12. All amounts and volumes indicated in **Subheadings 2.** and **3.** refer to a PCV of 1 mL usually obtained with $\sim 0.6 \times 10^9$ cells. We often find it more convenient to prepare more extract, and we usually start with a PCV of 2 mL obtained by pelleting $1\text{--}1.2 \times 10^9$ cells. In this case, all amounts and volumes should be doubled.
13. Rinse all glassware used during the cell extract preparation with PBS pH 7.6 just before use.

14. Add DTT to buffers (hypotonic lysis, sucrose-glycerol, and dialysis buffer) immediately before use. Some degradation products of DTT that form during prolonged storage may partially inactivate cell extracts.
15. In order to make active extracts, cells should be mycoplasma-free, healthy, and exponentially growing.
16. CHO-9 cells and its derivative mutants grow very well in Ham's F10/DMEM 1:1 supplemented with 10% fetal calf serum. Inoculate in each roller bottle a minimum of 30×10^6 cells.
17. The procedure for the in vitro repair assay is basically that described in Wood et al. (7,13) with minor modifications (5,8). The protocol described here refers to the use of plasmid DNA containing a single lesion as DNA substrate.
18. A simplified version of the in vitro BER assay involves the use of DNA substrates containing multiple AP sites (8). Briefly, for each reaction, 300 ng of heat-depurinated plasmid (e.g., pUC12, 2.7 kb) (see **Note 1**) are mixed with 300 ng of control undamaged plasmid of a different size (e.g., pBR322, 4.3 kb) and incubated with cell extracts as described in **Subheading 3.3.**, the only exception being the use of α - ^{32}P -dATP as the labeled deoxynucleotide. This is because a preferential loss of purines is produced after heating. The plasmid DNAs are purified by standard procedure and linearized with a restriction endonuclease that cuts at a single site. Damaged and undamaged plasmids are then separated overnight by 1% agarose-gel electrophoresis in the presence of ethidium bromide (0.5 mg/mL). Repair synthesis is quantified by densitometric scanning of the gel autoradiograph. The amount of α - ^{32}P -dAMP incorporated is corrected for the amount of recovered DNA as measured by densitometric scanning of the photographic negative of the gel.
19. Unless otherwise indicated, perform all operations in microcentrifuge tubes in racks on wet ice.
20. Two BER pathways have been identified in mammalian cells: a single-nucleotide insertion pathway, which is catalyzed by DNA polymerase β (9,14), and a PCNA-dependent pathway, which involves the resynthesis of 2-6 nucleotides 3' to the lesion (4-6). With pGEM-X, the use of α - ^{32}P -TTP allows one to monitor mainly short-patch BER (the single AP site is located opposite an adenine and one-gap filling reactions are the major repair route for this lesion), whereas α - ^{32}P -dCTP is suggested for measuring long-patch BER (cytosine is the most represented base 3' to the abasic site) (**Figs. 1 and 3**).
21. Better reproducibility can be obtained if fresh batches of α - ^{32}P -labeled deoxynucleotides are used. Stabilized aqueous solutions (such as the Redivue Amersham preparations) are also recommended.
22. Take care that all buffers (e.g., dialysis buffer in which extracts are dissolved) are present in equal amounts in all the reactions.
23. An incubation time of 1 h is usually appropriate for studying both the short- and long-patch BER pathways. The latter pathway is slower than the former, but both reactions are completed within 60 min. However, because of a certain degree of variability in the repair activity of different extract preparations, we suggest run-

ning a preliminary kinetic experiment (15-, 30-, 60-, and 180-min incubation times) when setting up the system. This kind of experiment is very informative and may help in choosing the most suitable incubation time.

24. At this step, the samples can be stored in a Plexiglas box at -80°C for 24–48 h if necessary.
25. In the case of pGEM-X, incubation of the repaired plasmid DNA with *Sma*I and *Hind*III restriction endonucleases releases a fragment of 33 bp, which contained originally the abasic site (**Fig. 3**). This restriction fragment can be easily analyzed by 15% denaturing polyacrylamide gel electrophoresis. The occurrence of complete repair is shown by the appearance on the gel autoradiograph of a band corresponding to a 33-mer. Unligated products containing one replaced nucleotide migrate as 16-bp oligonucleotides (**Fig. 3**). Appropriate DNA size markers can be obtained by 5'-end-labeling oligonucleotides having the same sequence as the restriction fragment.

Acknowledgments

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Nucleotide Excision Repair in *Saccharomyces cerevisiae* Whole-Cell Extracts

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1. Introduction

Nucleotide excision repair (NER) is a particularly versatile pathway of DNA repair capable of removing a broad spectrum of DNA lesions in both prokaryotes and eukaryotes (1–3). NER involves steps of damage recognition, incision and excision of the lesion and its flanking DNA, and repair DNA synthesis to fill in the resulting single-stranded gap. Here we describe the techniques used to prepare extracts from *Saccharomyces cerevisiae* cells capable of performing NER reactions and the details of this in vitro NER assay. Studies of DNA repair in *S. cerevisiae* have the advantage of being amenable to powerful genetic analyses within a completely sequenced yeast genome. In fact, most of the earlier work on NER in yeast relied on the genetic analyses of *rad* mutants. On the other hand, the potential for biochemical analysis of NER in yeast has not yet been fully realized owing in part to the lack of a simple in vitro repair system. This is in contrast to the situation in human cells in which the in vitro system developed by Wood and his colleagues (4; see Chapter 29) has proven instrumental in dissecting the human pathway of NER.

Thus far, two cell-free repair systems employing *S. cerevisiae* extracts have been described, both by Wang et al. from the laboratory of E. Friedberg (5,6). The first-described system utilizes a nuclear extract supplemented with whole-cell extract made from a yeast strain that overexpresses the Rad2 protein (5). The second system is simpler and makes use of yeast whole-cell extracts. However, this protocol requires the preparation of spheroplasts and same-day processing after the harvesting step (6). Here, we describe a simple in vitro system using whole-cell extract prepared from frozen *S. cerevisiae* cells (7). Some unique features of our system include ease of preparation and general appli-

cability for use with practically any yeast strain. The harvested yeast can be stored frozen at -70°C until the day of extract preparation. Moreover, this extract preparation can be used as starting material for further biochemical manipulations, such as antibody-depletion experiments to remove a particular protein component (7).

This procedure for the preparation of NER-proficient yeast extract is a modification of protocols originally used for studies of *in vitro* transcription by RNA polymerases (8). More recently, this same extract has also been shown to support chromatin assembly (9). The critical steps in our own protocol appear to be the freezing and grinding of yeast cells under liquid nitrogen to preserve cellular activities and minimize protease activity, as well as the high protein concentration achieved by ammonium sulfate precipitation. Further characterization of the activities of this extract is certainly worth pursuing, since it may provide a system for *in vitro* studies of additional pathways of DNA repair.

2. Materials

2.1. Equipment

1. Cryogenic and leather gloves.
2. Stainless-steel spatula: cool with dry ice before use.
3. Matched porcelain mortar and pestle, e.g., Coors #60319 (275 mL, 115×70 mm) and #60320 (VWR Canlab, Mississauga, Ontario). Store at -70°C the day before use.
4. Cryoflask for storing liquid nitrogen.
5. 50-mL sterile, disposable polypropylene tubes.
6. 4-L Erlenmeyer flasks.
7. Beakers: 1 L, 500 mL, and 250 mL.
8. Syringe: 20 mL, cooled in -20°C freezer before use.
9. Pasteur pipets.
10. Blotting paper: 0.33 mm (Grade 238, VWR Canlab) or equivalent.
11. Dialysis tubing: 12,000–14,000 mol-wt cutoff.
12. 1.5-mL microcentrifuge tubes.

2.2. Solutions and Reagents

1. Water: High-quality deionized water such as that provided from a Milli-Q (Millipore) system should be used in all procedures and for making up all solutions. Buffers should be made using autoclaved water or stock solutions.
2. Growth media: YPD (also known as YEPD, 1% yeast extract, 2% Bacto-Peptone, 2% glucose) (*see Note 1*).
3. Yeast Extraction Buffer: 0.2 M Tris-acetate, pH 7.5, 0.39 M $(\text{NH}_4)_2\text{SO}_4$, 10 mM MgSO_4 , 20% (v/v) glycerol, 1 mM EDTA. Store at 4°C . Add dithiothreitol (DTT) to 1 mM and protease inhibitors before use.
4. Protease inhibitor stocks (*see Notes 2 and 3*): 100 mM phenylmethylsulfonylfluoride (PMSF) (Sigma, Oakville, Ontario) in absolute ethanol (100X); 1 M

benzamidine hydrochloride (Sigma) in water (1000X); 3.5 mg/mL pepstatin A (Calbiochem, San Diego, CA) in DMSO (1000X); 1 mg/mL leupeptin (Calbiochem) in water (1000X); 0.35 mg/mL bestatin (Boehringer Mannheim, Laval, Quebec) in water (1000X); and 2 mg/mL aprotinin (Sigma) in water (200X). Store all stocks at -20°C .

5. Solid ammonium sulfate: analytical grade.
6. Dialysis buffer: 20 mM HEPES-KOH, pH 7.5, 20% (v/v) glycerol, 10 mM MgSO_4 , 10 mM EGTA, 5 mM DTT, and 1 mM PMSF. Cool in cold room ($4-10^{\circ}\text{C}$) before use.
7. Plasmid DNA: pUC18 (2.7 kbp) and pGEM-3Zf(+) (3.2 kbp pairs). Purify by CsCl centrifugation according to established protocols (**10**) or by any other procedures, such as with a QIAGEN DNA purification kit, that yield similarly high-quality DNA.
8. *N*-acetoxy-2-acetylaminofluorene (National Cancer Institute Chemical Carcinogen Repository, Kansas City, MO, cat.# AM0030): 1 mM in ethanol, store at -20°C .
9. dNTP stocks and ATP: ultrapure set (Pharmacia, Baie-d'urfé, Quebec): supplied as 100 mM solutions, pH 7.5.
10. 4X Repair buffer A: 180 mM HEPES-KOH, pH 7.8, 280 mM KCl, 29.6 mM MgCl_2 , 3.6 mM DTT, 1.6 mM EDTA, and 13.6% glycerol. Store at -20°C .
11. 4X Repair buffer B: 8 mM ATP, 80 μM dGTP, 80 μM dATP, 80 μM TTP, 32 μM dCTP. Store at -20°C .
12. 10 mg/mL Bovine serum albumin (BSA) (Sigma, Fraction V): Dissolve in water, and store in aliquots at -20°C .
13. Disodium phosphocreatine (Sigma): 1 M stock in water. Store at -20°C .
14. Creatine phosphokinase (Sigma): 2.5 mg/mL in 50 mM HEPES-KOH, pH 7.6, 20 mM magnesium acetate, and 50% glycerol. Store at -20°C .
15. [α - ^{32}P]dCTP (New England Nuclear, Mississauga, Ontario): SA of 3000 Ci/mmol.
16. 10% SDS: 10% (w/v) sodium dodecyl sulfate in water. Store at room temperature.
17. Proteinase K (Boehringer Mannheim): Make a 20 mg/mL stock in water. Store in 50- μL aliquots at -20°C .
18. Ribonuclease A (Sigma): 10 mg/mL in water. Boil for 10 min to inactivate DNase. Store in aliquots at -20°C .
19. Phenol/chloroform: Buffered-saturated phenol (Gibco BRL, Burlington, Ontario):chloroform:isoamyl alcohol (25:24:1). Phases are allowed to separate after vigorous shaking. Wrap in foil and store at room temperature.
20. 7.5 M ammonium acetate.
21. Ethanol: absolute (100%) and 70%.
22. Restriction enzyme *Hind*III (Gibco BRL): Store 10X buffer and enzyme in small aliquots at -20°C to ensure optimal activity.
23. 1X TBE: 89 mM Tris-base, 89 mM boric acid, and 2 mM EDTA, pH 8.0.
24. Agarose: Molecular biology grade.
25. 5 and 20% Sucrose: Dissolve ultrapure grade sucrose in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.5 M NaCl. Prepare fresh and cool to 4°C .

26. Ethidium bromide stock: Dissolve 10 mg/mL in water. Wrap in foil, and store at room temperature.
27. TE buffer: 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. Prepare using autoclaved stock solutions.

3. Methods

3.1. Yeast Growth and Harvesting

1. Prepare a starter culture by inoculating the yeast strain into 10 mL of growth media (YPD) in a 50-mL sterile disposable tube (*see Note 1*). Grow until saturation at 27°C with shaking (250 rpm).
2. Inoculate 2 L of YPD in a 4-L Erlenmeyer flask with the 10 mL starter culture. Incubate at 27°C with shaking overnight (24 h) (*see Note 4*).
3. Chill the flask in ice water. Transfer the culture to a 1-L centrifuge bottle (weigh the empty bottle; *see step 8*). Cultures should be kept cold during the entire harvesting procedure.
4. Harvest the cells by centrifugation at approx 4500g for 5 min at 4°C. For a Beckman J6-HC centrifuge fitted with a JS-4.2 rotor, the speed is 4000 rpm.
5. Discard the supernatant. Add any remaining culture and recentrifuge.
6. Wash the cells by thoroughly resuspending in 100 mL of ice-cold water. Pellet the cells by centrifugation as in **step 4**. Discard the supernatant.
7. Resuspend the cells in 100 mL of cold yeast extraction buffer with DTT and protease inhibitors (*see Notes 2 and 3*). Centrifuge as above.
8. Drain the supernatant. Remove residual supernatant with a pipet. The wet weight of cells can be determined at this step if the yield per liter of culture is of interest (*see Note 5*).
9. With the help of a spatula, scoop the yeast paste into a cooled 20-mL syringe with the plunger removed. Do not fit the syringe with a needle.
10. Extrude the yeast cells directly into a small plastic container (e.g., a 250-mL beaker) filled with liquid nitrogen (N₂). Keep filling the container with liquid nitrogen so that the yeast “noodles” are always completely submerged under N₂.
11. Break the yeast noodles into pieces with the help of a spatula or a pestle. This helps to keep the volume down and allows easier handling in future steps.
12. The frozen yeast can now be transferred to a -70°C freezer for long-term storage or until the day of extract preparation. The container should be covered with aluminum foil to allow liquid nitrogen to evaporate. Make sure the container is not tightly capped to avoid pressure buildup and explosion.

3.2. Preparation of Whole Cell Extract

1. Transfer the container with yeast (from **step 12** of **Subheading 3.1.**) from the freezer into a bucket of dry ice.
2. Weigh out the desired quantity of frozen yeast cells into a small beaker (precooled on dry ice) (*see Note 6*). Transfer onto dry ice immediately after weighing. All of the following steps are carried out in a cold room.

3. Take the cooled mortar and pestle to a cold room. Fill to three-quarter full with N_2 , which will evaporate away relatively quickly. Fill the mortar with N_2 again. (**Caution:** Liquid nitrogen is hazardous both for its extremely low temperature as well as the risk of asphyxiation if used in a small, unventilated room.)
4. Pour the weighed yeast noodles into the mortar.
5. Start crushing the frozen yeast under N_2 by stomping the noodle fragments gently against the bottom of the mortar with a pestle. Pressing the yeast against the side and bottom also helps. The goal is to reduce the noodles into even smaller chunks or pellets.
6. Replenish the N_2 if it has boiled off.
7. Begin the grinding step that will break the cells by driving the pestle in a circumferential path around the mortar, applying pressure against the side wall.
8. Every now and then, refill the mortar with N_2 . Wait until most of the N_2 has boiled off before initiating grinding. The optimal amount of N_2 is such that the yeast powder is just barely resuspended in it to form a slurry, with no obvious layer of excess N_2 above the yeast.
9. If some yeast powder sticks to the wall of the mortar, use the pestle to scrape it off to the bottom below the N_2 . Grind until the yeast shows a powdery, smooth consistency (*see Note 7*).
10. After the grinding step, pour more N_2 into the mortar, washing all the powder to the bottom with the aid of the pestle.
11. Pour the ground yeast/liquid nitrogen suspension into a 1-L plastic beaker previously cooled with N_2 . Scrape any remaining yeast powder into the beaker with a spatula.
12. The beaker with the ground yeast can now be covered with aluminum foil and transferred to a -70°C freezer for storage. Alternatively, if extract preparation is to be done right after the grinding step, let the N_2 boil off completely from the beaker in a cold room.
13. Add yeast extraction buffer (1 mL/1 g of yeast) supplemented with DTT and protease inhibitors. It is not necessary to precool the buffer (*see Note 8*).
14. Allow the yeast to thaw slowly in the cold room. It may take 15–20 min to obtain a fluid suspension. Disperse any clumps of powder by pipeting up and down.
15. Transfer the thawed extract to a centrifuge tube on ice. Centrifuge at 120,000g for 2 h at 4°C . For a Beckman type 70 Ti rotor, the speed would be 33,000 rpm.
16. After centrifugation, recover the clear portion of the supernatant using a Pasteur pipet (*see Note 9*). Transfer the material to a 250-mL beaker.
17. Measure the volume of the supernatant collected. Weigh out 337 mg solid ammonium sulfate/mL of lysate.
18. Add the solid ammonium sulfate in small portions over the course of 1 h with gentle continuous stirring by a small magnetic stir bar.
19. Stir the suspension for another 30 min after all the ammonium sulfate is added (*see Note 10*).
20. Recover the precipitated protein by centrifugation at 40,000g (approx 20,000 rpm in a 70 Ti rotor) for 15 min at 4°C .

21. Remove the supernatant. Resuspend the pellet in a small volume (approx 50 $\mu\text{L/g}$ of yeast) of dialysis buffer containing protease inhibitors (*see item 4 of Subheading 2.2.*).
22. Transfer the resuspended material to the dialysis tubing. Dialyze overnight (12–16 h) against 1 L of dialysis buffer.
23. After dialysis, recover the dialysate to a microcentrifuge tube, and centrifuge for 1–2 min to pellet any precipitated protein (*see Note 11*).
24. Recover the clarified supernatant. Freeze the sample in small aliquots in dry ice and store at -70°C (*see Note 12*).

3.3. Preparation of Damaged DNA Substrates

The procedures for preparing DNA repair substrates are essentially the same as described (6,11) and are outlined below in detail. Plasmid pGEM-3Zf(+) is used as the undamaged control whereas the smaller pUC18 is used to prepare AAAF-damaged DNA.

1. Incubate 100 μg of pUC18 at 30°C for 3 h in 1 mL of TE buffer containing 3 μM *N*-acetoxy-2-acetylaminofluorene (*see Note 13*). (**Caution:** AAAF is a potent carcinogen. Dispose of contaminated equipment according to safety guidelines.)
2. Layer the DNA sample onto a linear 5–20% sucrose gradient in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.5 M NaCl.
3. Centrifuge at 28,000 rpm in a Beckman SW41 rotor (135,000g) for 17 h at 4°C .
4. Collect fractions (each 0.5–1 mL) from the bottom of the centrifuge tube.
5. Check 3 μL of each fraction by electrophoresis on a 1% agarose gel cast in 1X TBE containing 0.5 $\mu\text{g/mL}$ ethidium bromide.
6. Identify the fractions containing closed circular, supercoiled DNA.
7. Pool these fractions and recover the DNA by ethanol precipitation.
8. Dissolve the DNA in TE buffer so that the DNA concentration is at least 100 ng/ μL . Store at -20°C .

3.4. In Vitro DNA Repair Synthesis

Nucleotide excision repair in the yeast cell extract is monitored by the incorporation of radiolabeled nucleotides into AAAF-modified plasmid DNA during repair DNA synthesis. An untreated plasmid is also included in each reaction to monitor damage-independent nucleotide incorporation. The assay is essentially an adaptation of the protocol developed for human cell extracts (4).

1. Quantitate the protein concentration in the extract (**Subheading 3.2.**) by Bradford assay (Bio-Rad Protein Assay) using BSA as standard (*see Note 14*).
2. To a 1.5-mL microcentrifuge tube, add 12.5 μL of 4X repair buffer A, 12.5 μL of 4X Repair Buffer B, 1.8 μL of 10 mg/mL BSA, 2 μL of disodium phosphocreatine, 1 μL of creatine phosphokinase, 300 ng each of AAAF-treated pUC18 and control pGEM-3Zf(+) DNA, and 2 μCi of [α - ^{32}P]dCTP (*see Note 15*).
3. Add 250 μg of yeast extract (typically 6–8 μL) and water to a final total volume of 50 μL . Mix gently (*see Note 16*).

4. Incubate at 28°C for 2 h.
5. Stop the reaction by adding 1 μL of 1 *M* EDTA. Add 0.5 μL of ribonuclease A, mix, and incubate at 37°C for 10 min.
6. Add 2.5 μL of 10% SDS and 0.5 μL of proteinase K. Mix and incubate at 37°C for 30 min.
7. Extract with an equal volume of phenol/chloroform. Centrifuge for 10 min at room temperature in a microcentrifuge.
8. Carefully transfer the upper aqueous phase to a new microcentrifuge tube. Repeat the extraction procedure one more time.
9. Add ammonium acetate to 2.5 *M* (1/2 vol of a 7.5 *M* stock) and precipitate the DNA with 2 vol of absolute ethanol at -70°C for a minimum of 10 min.
10. Recover the DNA by centrifugation in a microcentrifuge at top speed for 10 min. Remove the supernatant.
11. Add 100 μL of 70% ethanol. Vortex and centrifuge as above.
12. Allow residual ethanol to evaporate by leaving the tube open for at least 10 min.
13. Digest the DNA overnight with *Hind*III (20 U) in a 20- μL final volume.
14. Add gel-loading buffer, and subject the sample to electrophoresis on a 1% agarose gel plus ethidium bromide.
15. Photograph the gel under near-UV transillumination.
16. Transfer the gel to blotting paper, and vacuum dry at 80°C for 1 h.
17. Expose the dried gel to X-ray film to obtain a high-quality autoradiogram. Quantitation can be done by phosphorimaging analyses (*see Note 17*).
18. The result of a typical repair assay is shown in **Fig. 1** (*see Note 18*).

4. Notes

1. Yeast grown in rich medium, such as YPD, gives the highest yield. Usually 5 g/L can be obtained for a common laboratory strain, such as W303. However, if minimal medium, such as synthetic complete (SC) medium, must be used, a lower yield should be expected.
2. Other protease inhibitor cocktails can also be used (or supplemented) if it is believed to be necessary. PMSF is highly unstable in aqueous solution of pH > 7.0 and should be added to the buffer just before use. Consult a Boehringer Mannheim or other supplier catalog for a description of the specificities of these and other alternative protease inhibitors.
3. We have tried both protease-deficient and wild-type yeast strains (using identical conditions as described here), and they appear to yield similarly active extracts. The protocol described herein probably works for most, if not all strains (*see ref. 7 and Note 8*).
4. Growth of yeast can be monitored by measuring OD at 600 nm. We routinely carry out the harvest when the culture is grown to saturation. (OD of 2–4 for W303.) Exponential growth is not required to obtain an extract proficient in repair. If cells are cultured in SC medium, it will take longer (2 d at least) to get to saturation if the amount of starter-to-culture volume is kept at the same ratio, i.e., 10 mL to 2 L.

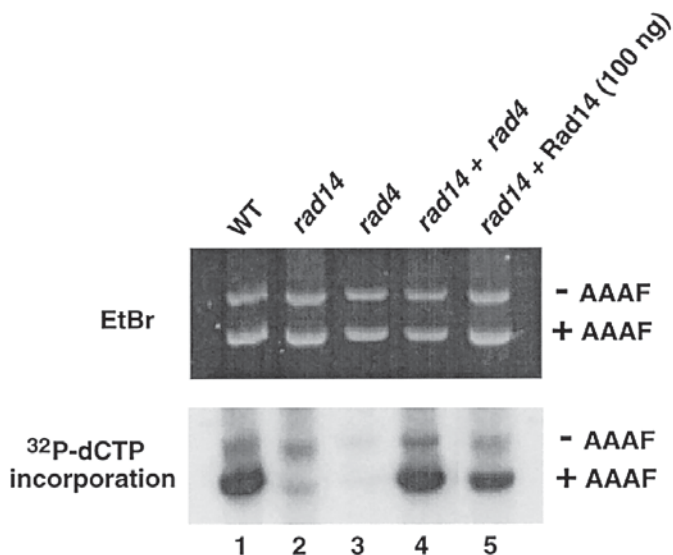


Fig. 1. Nucleotide excision repair in yeast whole-cell extracts. The repair activity was assayed by radioisotope incorporation into control or AAAF-treated DNA during repair DNA synthesis. The upper panel shows the DNA as visualized by ethidium bromide staining, and the lower panel shows the autoradiogram of the gel. Extracts prepared from *rad14* and *rad4* deletion strains exhibited only background levels of radiolabel incorporation (lanes 2 and 3). Mixing equal amounts of mutant extracts complemented the defects in excision repair (lane 4). Adding purified Rad14 protein also restored repair activity to the *rad14* extract (lane 5). See **Notes 17** and **18**.

5. Getting a rough idea of the yield of a particular strain in terms of yeast mass/L of medium may be helpful in future experiments. This allows one to calculate the minimum volume of cells needed to be grown to give, say, 10 g of yeast.
6. As little as 2 g of yeast can be processed. However, we recommend grinding 10 g of yeast, since this is the maximum amount the mortar can comfortably hold. This amount of starting material will eventually yield about 0.8–1 mL of concentrated extract.
7. At the initial phase of the grinding step, the consistency of the yeast can be described as “grainy” and “chunky,” since the material is essentially small pellets of frozen yeast. As grinding proceeds, the material turns from “grainy” or “sandy” into a evenly smooth powder that makes grinding more difficult. The powder will also tend to stick to the pestle and the side of the mortar. We estimate that roughly 300–400 circumferential passes around the mortar are sufficient to break 10 g of yeast. Total time required for just the grinding step is around 20–30 min with some rest periods.
8. The extraction buffer (room temperature) will immediately be frozen on contact with the frozen powder. Cells remaining frozen as they are broken and thawing

directly into a buffer containing protease inhibitors are likely the two important features contributing to the success of this protocol (*see* **Notes 2 and 3**).

9. Avoid the gummy material near the pellet of cellular debris at the bottom of the centrifuge tube. Also, avoid the white, loose layer of lipid frequently seen floating on the surface. As a result, up to 1/3 of the supernatant may not be retrievable. This ultracentrifugation step also helps remove yeast DNA.
10. Stirring should be regulated to keep frothing to a minimum. Frothing may promote denaturation and oxidation of proteins. This step of ammonium sulfate precipitation serves to concentrate protein and remove residual yeast DNA (*see* **Note 9**). However, some yeast proteins will not be precipitated, and therefore, the extract preparation is, after this step, not strictly speaking a whole-cell extract.
11. The amount of precipitation appears to vary for unknown reasons. However, the precipitation that occurs during dialysis does not seem to affect activity and may only reduce the final protein concentration.
12. Extracts prepared according to this protocol appear to lose some repair activity after multiple freezings and thawings. Therefore, aliquoting the extract into small fractions is highly recommended.
13. *N*-acetoxy-2-acetylaminofluorene forms DNA adducts known to be corrected only by nucleotide excision repair. UV-irradiated plasmid DNA, sometimes used as substrate for NER, is also known to be acted on by base excision repair (**11**).
14. The protein concentration of a typical extract preparation is usually around 25–35 $\mu\text{g}/\mu\text{L}$.
15. Optimal repair activity requires disodium phosphocreatine and phosphocreatine kinase, which constitute an ATP-regenerating system. The repair activity is also dependent on ATP and Mg^{2+} (**7**).
16. The extent of damage-dependent repair synthesis increases with the amount of extract protein added (**7**). An amount of extract corresponding to 250 μg of protein usually produces good signals above the background level of radioisotope incorporation into the control plasmid.
17. The amount of radioactivity in each band can be quantitated by phosphorimaging analysis and comparison to known radioactivity standards which are simultaneously exposed to the same phosphorimaging screen. Our assay routinely supports incorporation of 130–190 fmol of dCMP into the AAAF-treated DNA with background incorporation of 20–40 fmol into the untreated control DNA. This is comparable to that reported by Wang et al. (**5**) and is nearly as active as the human cell extracts described by Wood et al. (**4**).
18. Note that incorporation of labeled nucleotides into AAAF-treated plasmid is dependent on *RAD* genes, such as *RAD14* and *RAD4* (**Fig. 1**, lanes 2, and 3). A negative control using a *rad* mutant extract should always be included in every experiment. We also recommend performing a complementation experiment by mixing two appropriate mutant extracts (e.g., *rad4* and *rad14*) in order to confirm that the incorporation does represent bona fide excision repair activity (**Fig. 1**, lane 4).

Acknowledgments

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In Vitro Excision Repair Assay in *Schizosaccharomyces pombe*

Bernard Salles and Patrick Calsou

1. Introduction

The measurement of DNA excision repair activity in vitro, originally developed by Wood and co-workers (*1*), utilizes transcriptionally active protein extracts obtained from mammalian cells by the method of Manley et al. (*2*) (see Chapter 29). Nucleotide excision repair (NER), which requires more than 20 proteins in vitro, can process a large variety of lesions according to the following mechanism:

1. Recognition of the DNA lesion.
2. Incision on both sides of the lesion.
3. Excision of the damaged oligonucleotide.
4. DNA polymerization and ligation.

The repair assay relies on the incorporation of a radiolabeled deoxynucleotide during the repair synthesis step. In order to observe repair-replication, supercoiled plasmid DNA damaged, for example, by UV-C light, is incubated with whole-cell extract in a reaction mixture including the four dNTPs, one of them radiolabeled, ATP, and an ATP-regenerating system. Undamaged plasmid DNA of a slightly different size is added to the reaction mixture as a control. DNA repair synthesis is determined after recovery of plasmid DNA from the mixture, linearization with a restriction enzyme, agarose-gel electrophoresis, autoradiography and measurement of the radioactivity incorporated into each plasmid (*1*). NER activity can be expressed either as specific repair synthesis (incorporation in the damaged plasmid minus background incorporation in the control plasmid) or as a relative repair factor (ratio of incorporation in damaged vs control plasmid). When radioactive incorpora-

tion is observed in plasmid without linearization, the radioactivity associated with repair events is mainly recovered in the closed-circular form, indicating that the reaction proceeds through to the ligation step. The extent of completion of the repair reaction can be determined by visualization of the closed-circular and nicked-circular forms and measurement of their respective radioactive contents (3–5).

During incubation, DNA lesions are removed by NER but also perhaps by base excision repair (BER). BER consists of the following steps:

1. Recognition of a narrow spectrum of base damage by a specific DNA glycosylase and cleavage of the *N*-glycosidic bond resulting in an apurinic/apyrimidinic (AP) site.
2. Excision of the AP site by cleavage of the sugar-phosphate DNA backbone.
3. Repair synthesis of a short patch and DNA ligation to restore strand continuity (see Chapters 22–24).

The extent of repair by BER vs NER depends not only on the chemical nature of the DNA damage (6), but also on the level of limiting protein(s), which in turn depends on the method of extract preparation. This point is illustrated by the observation that nuclear extracts from *Saccharomyces cerevisiae* can perform the NER reaction in vitro only when supplemented with the rate-limiting Rad2 protein (7,8, but see Chapter 25).

Although the genetics and molecular biology of DNA repair and mutagenesis have been more widely studied in *S. cerevisiae* than in *Schizosaccharomyces pombe*, there is a growing interest in the fission yeast. In terms of repair activity, *S. pombe* is more resistant to UV light compared to *S. cerevisiae*. This has led to a concept of multiple repair pathways for UV damage: UV light provokes the formation of DNA photoproducts that can be excised by both NER and BER pathways in *S. pombe* (9–11).

The extent and specificity of repair synthesis are highly dependent on the quality of cell-free extract and plasmid DNA as well as the type/extent of DNA damage and the reaction conditions (e.g., salt concentration, pH). We have tested various extraction procedures (e.g., total cellular vs nuclear protein extracts). However, in our hands these different extracts give similar repair synthesis activities, and for convenience, we use a total cellular extract to assay excision repair activity in *S. pombe* (12). It is clear that the repair signal in this assay is owing to BER and not NER, since cisplatin-induced damage, which is normally a substrate for the NER complex (see Chapter 30) is not repaired in vitro. The absence of NER activity in *S. pombe* extract is likely to result from the lack of Rad13, the homolog of Rad2 found in *S. cerevisiae* (11). Excision repair is also found to be inducible when *S. pombe* is irradiated with UV-C before preparation of the cell extract (12; Fig. 1).

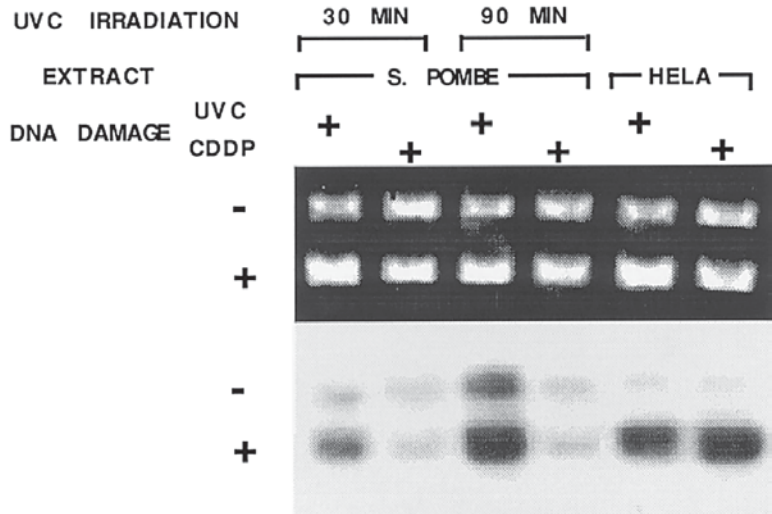


Fig. 1. *S. pombe* suspension was UV-C irradiated and cell extracts were prepared 30 and 90 min postirradiation as described; 150 μ g of protein extract were incubated in the presence of undamaged and damaged plasmid DNA (either by UV-C or cisplatin [CDDP]). Top panel: ethidium bromide-stained gel showing migration of undamaged control plasmid (pHM14, top bands) and damaged pBS (bottom bands). Bottom panel: autoradiographic image of gel showing radiolabel incorporation. A repair synthesis signal is observed in UV-C-damaged (lanes 1 and 3), but not in the CDDP-damaged (lanes 2 and 4) DNA. The control reaction with HeLa cell extracts (100 μ g) shows that CDDP lesions are repaired to approximately the same extent as UV-C photoproducts (lanes 5 and 6). The *S. pombe* extract is proficient at base excision repair, but not nucleotide excision repair. An induction of repair synthesis is observed in the case of UV-C-damaged plasmid (compare lanes 1 and 3) (see also ref. 12).

2. Materials

2.1. Plasmid Preparation

The use of purified closed-circular plasmid DNA is important, since the presence of nicked plasmid DNA constitutes a potential source of 3'-OH primers used in the polymerization reaction to quantify repair activity synthesis.

1. *Escherichia coli* JM109 strain (relevant genotype: *recA1*, *endA1*, *gyrA96*, *hsdR17*; see Note 1) transformed with the 2959 bp plasmid pBluescript KS⁺ (pBS; Stratagene, LTD, Cambridge, UK) or the related 3738 bp pHM14 plasmid (gift from R. D. Wood, ICRF, UK).
2. Culture medium (Luria broth): 10 g/L Bacto-tryptone, 5 g/L yeast extract, 8 g/L NaCl.

3. Reagents for plasmid preparation by alkaline lysis (**13**) or Qiagen plasmid isolation kit (Qiagen, St. Courtaboeuf, France).
4. Plasmid recovery:
 - a. TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
 - b. CPI: chloroform/phenol/isoamyl alcohol (25:24:1, v/v).
 - c. Ethanol (100 and 70%) or isopropanol.
 - d. 10 M Ammonium acetate.
 - e. Cesium chloride (CsCl) as powder.
 - f. Ethidium bromide, 10 mg/mL in water.
 - g. Butanol saturated with TE.
 - h. Sucrose (UltraPure™; Gibco BRL, Life Technologies SARL, Lugy Pontoise, France) for a 5–20% gradient.

2.2. DNA-Damaging Treatment

1. UV-C (254 nm) light source and UV-C dosimeter.
2. Cisplatin (*cis*-dichloro-diammine Pt[II]dichloride; CDDP) solution: 0.5 mg/mL cisplatin in 150 mM KCl. Store in aliquots at –20°C. All cisplatin solutions should be protected from light.
3. 5 M NaCl.

2.3. Yeast Extract Preparation

1. Yeast pellet (*see Note 2*): *S. pombe* strain grown in YE medium (0.5% yeast extract 3%, dextrose, 50 µg/mL adenine).
2. Glass beads (425–600 µm, Sigma-Aldrichchimic SARL, St. Quentin Fallavier, France).
3. Buffer A: 10 mM Tris-HCl, 10 mM MgCl₂, 10 mM KCl, pH 7.5. Store in aliquots at –20°C.
4. Protease inhibitor stock solutions (store in aliquots at –20°C): Aprotinin (4 mg/mL in 10 mM HEPES, pH 8.0); chymostatin (3 mg/mL in DMSO); pepstatin (1.5 mg/mL in 70% ethanol); leupeptin (3 mg/mL in H₂O); phenylmethylsulfonyl fluoride (PMSF, 17.4 mg/mL in isopropanol); N α -*p*-tosyl-L-lysine chloromethyl ketone (TLCK; 1 mg/mL in sodium acetate, pH 5.0); N-tosyl-L-phenylalanine chloromethyl ketone (TPCK; 3 mg/mL in ethanol).
5. Buffer A* (prepare fresh on the day of the protein extract preparation):
 - a. 10 mL of buffer A.
 - b. 50 µL of 1 M dithiothreitol (DTT).
 - c. 5 µL each of chymostatin and leupeptin.
 - d. 10 µL each of aprotinin and pepstatin.
 - e. 20 µL each of TPCK and TLCK.
 - f. 50 µL of PMSF.
6. Saturated ammonium sulfate solution, pH 7.5, 4°C.
7. Buffer B: 50 mM Tris-HCl, 10 mM EDTA, 100 mM KCl, 25% sucrose, 50% glycerol, pH 7.5. Store in aliquots at –20°C.
8. Ammonium sulfate powder (finely ground with a pestle and mortar).

9. 1 M NaOH.
10. Dialysis buffer: 25 mM HEPES-KOH, 0.5 mM EDTA, 17% glycerol, 2 mM DTT, 100 mM potassium glutamate, pH 7.8.

2.4. Excision Repair Reaction

1. Repair (*see Note 3*):
 - a. 5X buffer: 220 mM HEPES-KOH, pH 7.8, 35 mM MgCl₂, 2.5 mM DTT, 100 μ M dNTPs, except that used to label the reaction (20 μ M), 200 mM phosphocreatine, 17% glycerol, 1.5 mg/mL bovine serum albumin (BSA).
 - b. 3 M Potassium glutamate.
 - c. 1 M ATP.
 - d. 2.5 mg/mL creatine phosphokinase.
 - e. 74 kBq of [α -³²P]dATP or dCTP (110 TBq/mmol).
 - f. Cell extract kept frozen.
 - g. Dialysis buffer (*see item 10, Subheading 2.3.*).
2. Plasmid purification:
 - a. 500 mM EDTA, pH 8.0.
 - b. 10% Sodium dodecyl sulfate (SDS).
 - c. 20 mg/mL proteinase K.
 - d. CPI (*see item 4, Subheading 2.1.*).
 - e. 10 M Ammonium acetate.
 - f. Ethanol, 70 and 100%.
 - g. TE (*see item 4, Subheading 2.1.*).
 - h. 2 mg/mL RNase A.
3. Restriction enzyme and buffer.

3. Methods

3.1. Plasmid Purification (*see Note 4*)

1. Prepare plasmid DNA by standard alkaline lysis of bacteria (**13**) or by using a Qiagen plasmid DNA isolation kit following the manufacturer's instructions.
2. Dissolve the plasmid DNA pellet in TE.
3. Run a CsCl gradient (1 g/mL) in the presence of ethidium bromide (1 mg/mL). Centrifuge at 42,000 rpm in a Ti 50 rotor (160,000g) for 48 h at 20°C (*see Note 5*).
4. Recover the lower plasmid band, remove the ethidium bromide by extraction with butanol three to four times, precipitate and centrifuge the plasmid DNA, and dry the pellet. (A detailed protocol for a similar extraction procedure is given in Chapter 29.) Dissolve the pellet in TE such that 500 μ g of DNA is resuspended in <1 mL of TE. Measure the DNA concentration by UV absorption (260 nm).
5. Run a sucrose gradient: Layer the plasmid DNA (500 μ g/40-mL tube) on top of a 5–20% sucrose gradient (*see Note 6*). Centrifuge at 25,000 rpm in a SW 28 rotor (113,000g) for 17 h at 2°C. (*See also* Chapter 29.)
6. After centrifugation, collect 40 1-mL fractions.
7. Detect the plasmid DNA by gel electrophoresis. To a 10- μ L sample of each fraction, add 2 μ L of sucrose/EDTA solution (50% sucrose, 50 mM EDTA), mix, and

electrophoresis on a 0.8% agarose gel containing 0.25% ethidium bromide. Pool the fractions containing the closed-circular form. In the case of pBS, this is normally 4 fractions, #22–25. Quantify the plasmid DNA concentration by UV absorption. Store at -70°C .

3.2. Plasmid-Damaging Treatments

3.2.1. UV-C Irradiation

1. Disperse 50- μL drops of plasmid DNA (50 $\mu\text{g/mL}$) in a Petri dish on ice.
2. UV-C irradiate the DNA at the desired dose (e.g., 300 J/m^2 ; determined with a dosimeter).
3. Pool the drops, and store in aliquots at -70°C (see **Note 7**).

3.2.2. Cisplatin

1. Add the cisplatin solution to the plasmid DNA (100 $\mu\text{g/mL}$) in TE (see **Note 8**).
2. Incubate in the dark at 37°C for 12 h.
3. Stop the reaction by the addition of NaCl to a final concentration of 0.5 M.
4. Precipitate the plasmid DNA, dry the DNA pellet, and dissolve in TE.
5. Measure the DNA concentration, and store in aliquots at -70°C . The extent of plasmid modification can be verified by spectroscopic atomic absorption.

For details of cisplatin adducts and platination reactions see Chapter 30.

3.3. Preparation of Yeast Extracts (see **Note 9**)

1. Grow *S. pombe* to a density of approx 2×10^7 cells/mL in YE medium.
2. Harvest the cells by centrifugation at 1800g for 10 min, and wash the pellet twice with water.
3. In the case of UV-C irradiation, resuspend the pellet in PBS (2×10^7 cells/mL), and irradiate the yeast at the desired dose with gentle stirring in a Petri dish (depth of the cell suspension should be 1–2 mm).
4. Harvest the cells by centrifugation as in **step 2**, and incubate in YE medium (2×10^7 cells/mL for the desired time).
5. Measure the packed cell volume (PCV). The following purification steps are performed at 4°C .
6. Add 4 PCV of buffer A* to the pellet in a test tube (10- or 30-mL).
7. Add glass beads (~4 PCV) to the test tube. Keep on ice for 20 min.
8. Disrupt the yeast by vortexing 5 times (at high speed), 1 min each. Cell disruption can be monitored by visual inspection using a microscope.
9. Transfer to a beaker, and slowly add 4 PCV of buffer B, followed by 1 PCV of saturated ammonium sulfate (pH 7.5) on a magnetic stirrer (~60 rpm).
10. Continue to stir for 30 min.
11. Centrifuge at 42,000 rpm in a SW 50 rotor (212,000g) for 3 h at 2°C .
12. Pipet the supernatant (normally 3–3.5 mL/5 mL of centrifuged solution) into a clean beaker.
13. Slowly add 0.33 g of solid ammonium sulfate/mL of solution.

14. Add 10 μL of 1 *M* NaOH/g of added ammonium sulfate.
15. Stir for 30 min.
16. Centrifuge at 10,000*g* for 20 min.
17. Discard the supernatant, and resuspend the protein pellet in dialysis buffer (~300 μL /1 mL of PCV).
18. Dialyze overnight at 4°C.
19. Determine the protein concentration (*see* **Note 10**).

3.4. Repair Assay

The repair reaction protocol is that reported by Wood et al. (1).

3.4.1. Repair Reaction

1. Prepare the following mixture per tube:
 - a. 10 μL of 5X buffer.
 - b. 1 μL of 1 *M* ATP stock diluted 10-fold.
 - c. 1 μL of Creatine phosphokinase.
 - d. 300 ng of undamaged plasmid (3 μL of a 100 $\mu\text{g}/\text{mL}$ solution).
 - e. 300 ng of damaged plasmid DNA.
 - f. Potassium glutamate to a final concentration of 70 *mM*.
 - g. 0.2 μL of radiolabeled dATP or dCTP.
 - h. Water to a 50- μL final volume (including the volume of extract to be added at **step 4**).
2. Dispense the reaction mixture to Eppendorf tubes.
3. When different extracts are to be tested, add dialysis buffer to compensate for variations in cell extract volumes (protein amount is constant).
4. Add cell extract (<20 μL , 100–200 μg of protein).
5. Incubate at 30°C for the desired period (standard reaction time is 3 h).

3.4.2. Plasmid Recovery

1. Stop the reaction by adding per tube:
 - a. 2 μL of 0.5 *M* EDTA.
 - b. 1.5 μL of 10% SDS.
 - c. 1.5 μL of Proteinase K.
2. Incubate for 20 min at 45°C.
3. Add 60 μL of CPI, vortex, and keep on ice for 5 min (*see* **Note 11**).
4. Centrifuge at 10,000 rpm (8000*g*) for 3 min (2°C), and then remove 45 μL of supernatant.
5. Add 45 μL of CPI, vortex, centrifuge, and remove 35 μL of supernatant.
6. Precipitate the plasmid with 2¹/₂ vol of 100% ethanol. Centrifuge.
7. Rinse the pellet with 70% ethanol.
8. Dry and resuspend the DNA in TE.
9. Linearize with restriction enzyme, and separate the products by agarose-gel electrophoresis (*see* **Fig. 1**).

3.4.3. Quantitative Determination of Radioactivity Incorporation

1. Normalize the plasmid DNA recovery in each reaction sample by densitometry of a photographic negative of the ethidium bromide-stained gel.
2. Measure the incorporation of labeled deoxynucleotide in each plasmid (control and damaged) by scintillation counting of excised DNA bands or using a phosphorimager.

4. Notes

1. *recA* and *gyrA* mutations are used to impede recombination and multimerization of the plasmid DNA; *endA* reduces the nuclease activity.
2. Yeast pellet can be kept at -70°C for several months prior to protein extraction and purification, although we prefer to process fresh pellets.
3. The reaction buffer (5X) can be kept for at least 3 mo at -20°C and can also contain 250 $\mu\text{g/mL}$ creatine phosphokinase. ATP solution is stored at -70°C , and sterilized potassium glutamate at 4°C .
4. 500 mL of bacterial culture normally yields 1 mg of plasmid DNA following CsCl gradient centrifugation and ~ 0.6 mg after sucrose gradient centrifugation.
5. Before the ultracentrifugation step, remove the protein precipitate by centrifuging the plasmid DNA in TE plus CsCl and ethidium bromide at 8000g for 5 min. Transfer the supernatant to the ultracentrifuge tubes.
6. Make up fresh solutions of 5, 10, 15, and 20% sucrose in the following buffer: 1 M NaCl, 25 mM Tris-HCl, 5 mM EDTA, pH 7.5. To a 40-mL centrifuge tube, gently add on the lip 9.5 mL of each sucrose solution in order of decreasing concentration. Store the gradient for at least 1 h at 4°C before use.
7. In addition to the formation of pyrimidine dimers and 6-4 photoproducts, UV-C irradiation causes DNA strand breaks and base oxidation. To remove oxidized bases, plasmid DNA can be treated with *E. coli* endonuclease III (*Nth* protein) before sucrose gradient centrifugation (*see* Chapter 29). Since we do not observe differences in the repair signal with a 300 J/m² UV-C dose when the plasmid is treated with endonuclease III or otherwise, we do not use this treatment.
8. The final concentration of cisplatin in the reaction is calculated from the molar concentration of nucleotides, based on our knowledge that about 50% of the drug reacts with plasmid DNA.
9. Another procedure to prepare whole-cell extract has recently been described (14). *See also* Chapter 25.
10. The protein concentration should be >10 mg/mL. Low protein concentrations necessitate high extract volumes, which lead to partial inhibition of the repair reaction and incomplete repair activity.
11. If plasmid is not to be linearized at the end of the experiment (for example, in assays of the incision reaction [5]), vortexing should be avoided and gentle mixing performed instead.

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Nucleotide Excision Repair Assay in *Drosophila melanogaster* Using Established Cell Lines

Kenji Kohno and Takuya Shimamoto

1. Introduction

Nucleotide excision repair (NER) is one of the most important systems in eukaryotes for overcoming DNA damage caused by environmental agents, such as ultraviolet (UV) radiation and chemical mutagens. NER in eukaryotes has been studied by genetic analyses of mainly human, rodent, and yeast repair-deficient mutants (**1,2**). In particular, cultured cells derived from patients with xeroderma pigmentosum (XP), a human autosomal-recessive disease causing defects in NER, as well as from rodent excision repair crosscomplementing (ERCC) mutants have contributed to the elucidation of the molecular mechanisms of excision repair. Complementation analyses by cell fusion between cells from XP patients have defined seven complementation groups (A–G) and one variant (XP-V). With the exception of *XPV* and possibly *XPE* (*see* Chapters 7 and 9), these genes have been cloned and their counterparts from rodent and yeast identified (**1,2**). Elucidation of the molecular functions and interactions of these gene products have been advanced by the use of effective and powerful cell-free reconstitution systems. Wood et al. have developed a cell-free system utilizing UV-irradiated plasmid DNA and human cell extracts in which both UV-dependent NER and the differences between wild-type and XP cells are reproduced (**3**; *see* Chapter 29). Functional complementation between cell extracts in this in vitro system has proven effective in the isolation and identification of new factors involved in NER (**4**). Using a system employing SV40 minichromosomes instead of naked plasmid DNA in order to mimic the structure of DNA in vivo (**5,6**), Masutani et al. identified XPC and the human homolog of yeast RAD23 (**7**).

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We have cloned the *Drosophila* homolog of the human XPA gene (*Dxpa*) and found that it encodes a protein of 296 amino acids with 45% identity to its human counterpart (8). To characterize the Dxpa protein in *Drosophila* NER, we developed a *Drosophila* cell-free NER system prepared from cell extracts of cultured Kc cells and utilizing UV-irradiated SV40 minichromosome (9). This chapter describes the following detailed protocols for setting up the *Drosophila* NER assay system using cultured cell extracts:

1. Culturing of *Drosophila* Kc cell lines.
2. Preparation of whole cell extracts.
3. In vitro DNA repair assay.

2. Materials

2.1. Culturing of *Drosophila* Kc Cell Lines

1. Cells: *Drosophila* Kc cells (10) can be obtained from any of the groups working in this area or from the Mitsubishi Kagaku Institute of Life Science, Machida, Tokyo, 194 Japan (send requests to T. Miyake or R. Ueda). Kc cells were derived from *Drosophila melanogaster* embryos.
2. Medium: The composition of Modified M3(BF) medium is shown in **Table 1**. Dissolve all components listed in **Table 1** in about 950 mL of Milli Q-grade water (Millipore), and adjust to pH 6.8 with 1% NaOH. Make up to 1000 mL, and sterilize the medium by membrane filtration (0.22 μ m, Millipore). The medium should be stored at 4°C.
3. Fetal bovine serum (FBS): Commercially supplied FBS is heat-inactivated at 56°C for 30 min. One volume of heat-inactivated FBS is mixed with 9 vol of modified M3(BF) medium. After heat inactivation, store FBS at 4°C.
4. 150-mm Plastic culture dishes (Corning, #430597).

2.2. Preparation of Whole-Cell Extracts

1. Phosphate-buffered saline (–): Dulbecco's phosphate-buffered saline (PBS[–]; pH 7.4) without CaCl₂ and MgCl₂. PBS(–) is sterilized by autoclaving.
2. Buffer A: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5 mM dithiothreitol (DTT). Add DTT just before use. Store at 4°C.
3. 0.2 M Phenylmethylsulfonyl fluoride (PMSF; Sigma): Dissolve 0.35 g of PMSF in 10 mL of ethanol. Store at –20°C.
4. 10 mg/mL Leupeptin (Sigma): Dissolve in sterile water. Store at –20°C.
5. 1 mg/mL Pepstatin (Sigma): Dissolve in dimethyl sulfoxide (DMSO). Store at –20°C.
6. 1 mg/mL Chymostatin (Sigma): Dissolve in DMSO. Store at –20°C.
7. Buffer B: 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 25% sucrose, 50% glycerol, and 2 mM DTT. Add DTT just before use. Store at 4°C.
8. Saturated (NH₄)₂SO₄: Dissolve 80 g of ammonium sulfate in 100 mL of sterile water at 80°C, and cool to room temperature.

Table 1
M3(BF) Medium^a

Contents	Concentration, g/L
MgSO ₄ · 7H ₂ O	4.4
CaCl ₂ · 2H ₂ O ^b	1.0
K · glutamate, H ₂ O	8.9
Na · glutamate	6.5
NaH ₂ PO ₄ · 2H ₂ O	0.9
Glucose	10.0
Oxaloacetic acid	0.25
Trizma base (Sigma) ^b	1.1
TC Yeastolate (Difco)	1.0
Aspartic acid	0.3
Threonine	0.5
Serine	0.35
Asparagine	0.35
Glutamine	0.6
Proline	0.4
Glycine	0.5
α-Alanine	1.5
Valine	0.4
Methionine	0.25
Iso-leucine	0.25
Leucine	0.4
Tyrosine	0.25
Phenylalanine	0.25
β-Alanine	0.25
Histidine	0.55
Tryptophan	0.1
Arginine	0.5
Lysine · HCl	0.85
Cysteine · HCl	0.2
Choline · Cl	0.05
Penicillin G (potassium salt)	0.03
Streptomycin sulfate	0.1

^aDissolve in about 950 mL of doubly distilled H₂O, adjust the pH to 6.8 with 1% NaOH. Make up to 1000 mL, and sterilize by Millipore filtration.

^bModifications of the original M3(BF) medium described by Cross and Sang (**11**).

9. Buffer C: 25 mM HEPES-KOH, pH 7.9, 0.1 M KCl, 12 mM MgCl₂, 1 mM EDTA, 17% glycerol, and 2 mM DTT. Add DTT just before use. Store at 4°C.
10. Glass Dounce homogenizer: Approximately 15-mL capacity with a B-pestle (Wheaton).
11. 5-mL Polyallomer tubes (Beckman).

2.3. *In Vitro* DNA Repair Assay

1. 0.5 M Creatine phosphate (Tris salt, pH 7.7).
2. ATP (Boehringer Mannheim): 100 mM, pH 7.0, ATP solution (RNase-free). Store at -20°C.
3. dNTP-C mixture: 3.33 mM each of dATP, dGTP, TTP. Prepare by 30-fold dilution of each 100 mM stock solution (Takara Biomedicals, Obsu, Japan) with sterile water. Store at -20°C.
4. dCTP (Takara Biomedicals, Obsu, Japan). Store at -20°C.
5. [α -³²P]dCTP (Amersham): 110 TBq/mmol (3000 Ci/mmol). Store at -20°C.
6. 100X MgCl₂-DTT solution: 700 mM MgCl₂, 100 mM DTT. Store at -20°C.
7. 10 mg/mL Phosphocreatine kinase (Type I, Sigma). Store at -20°C.
8. Bovine serum albumin (BSA; Takara #2320; DNase- and RNase-free grade). Store at -20°C.
9. pUC19 RF1 DNA: Prepare plasmids by the alkaline-lysis method, and purify by two consecutive cesium chloride-ethidium bromide equilibrium density gradient centrifugation steps as described by Sambrook et al. (12). The purified plasmids are mostly in the supercoiled form. Store at -80°C in small aliquots.
10. SV40 minichromosomes: Prepare SV40 virions as described by Lebowitz et al. (13). The SV40 virion suspension is adjusted to 0.1 M glycine-NaOH, pH 9.8, and 3 mM DTT, incubated at 37°C for 5 min, and then neutralized by the addition of 1/8 vol of 1 M Tris-HCl, pH 6.8. Store at -80°C in small aliquots.
11. UV irradiation: The SV40 minichromosome solution (0.3 mg/mL of viral DNA) is aliquoted into a 48-well plate on ice, and irradiated with a 254-nm germicidal UV light at a fluence rate of 1.5 W/m² with occasional stirring. The UV fluence rate is measured with a UV radiometer (Topcon).
12. 0.5 M EDTA, pH 8.0: Prepare disodium ethylenediaminetetraacetate-2H₂O as described by Sambrook et al. (12). Store at room temperature.
13. 1 mg/mL RNase A: Dissolve pancreatic RNase (RNase A, type IIA, Sigma) at a concentration of 1 mg/mL in 10 mM Tris-HCl, pH 7.5, 15 mM NaCl. Heat to 100°C for 15 min. Allow to cool slowly to room temperature. Dispense into aliquots, and store at -20°C.
14. Stop solution I (per tube): 2.5 μ L of 10% sodium dodecyl sulfate (SDS), 1 μ L of 20 mg/mL glycogen, 1 μ L of 10 mg/mL proteinase K, and 23.5 μ L of sterile water. Store at -20°C in aliquots.
15. Proteinase K (Boehringer Mannheim): Prepare a stock solution of 10 mg/mL in sterile water, divide into small aliquots, and keep frozen at -20°C.
16. Phenol-chloroform (1:1, v/v): Mix equal amounts of reagent-grade phenol and chloroform. Equilibrate the mixture with 10 mM Tris-HCl, pH 7.4. Store at 4°C (12).

17. 5 M Ammonium acetate: Prepare from reagent-grade ammonium acetate as described (12). Store at room temperature.
18. 10 mg/mL tRNA (Gibco BRL): An aqueous solution is made and extracted twice with phenol-chloroform. The RNA is precipitated with ethanol, pelleted down, dried, and redissolved in 10T1E to a concentration of 10 mg/mL. Store at -20°C in aliquots.
19. 10T1E: 10 mM Tris-HCl, pH 7.4, 1 mM EDTA.
20. *Eco*RI reaction mixture: 2 μL of 10X high-salt buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2 , 1 mM DTT), 0.5 μL of *Eco*RI (40 U/ μL ; Takara Biomedicals, Japan), and 2.5 μL of sterile water.
21. Stop solution II: 0.25% bromophenol blue, 0.25% xylene cyanol, 1 mM EDTA, 30% glycerol. Store at -20°C in aliquots.
22. TAE buffer: 40 mM Tris-acetate, 1 mM EDTA, pH 7.5. Prepare a 50X stock solution, adjusting the pH with glacial acetic acid. Store at room temperature.
23. X-ray film (e.g., Fuji New RX) and intensifying screen (e.g., Kodak X-Omat).

3. Methods

3.1. Culture of *Drosophila* Kc Cell Lines

1. Grow Kc cells in M3(BF) medium at 25°C in 150-mm plastic dishes under normal atmosphere (100% air). To maintain saturated humidity, the plastic dishes should be sealed by vinyl tape or placed in a tray filled with water at the bottom of the incubator.
2. Harvest late-log-phase cells by pipeting, and seed one-tenth the number to new dishes. Cells should be transferred every 3 or 4 d (see Note 1).

3.2. Preparation of Whole-Cell Extracts (14)

1. Grow Kc cells to a late-log phase (90% confluence) in 20 150-mm plastic dishes. The following operations should be carried out at $0-4^{\circ}\text{C}$.
2. Harvest cells from the dishes by pipeting or using a cell scraper.
3. Collect the cells in a 50-mL polypropylene tube by low-speed centrifugation (1800 rpm, $\sim 500g$) for 10 min. Determine the packed cell volume (PCV). The PCV should be about 2.5 mL ($5-10 \times 10^8$ cells).
4. Remove the medium and wash the cells twice with 20 mL of ice-cold PBS(-).
5. Resuspend the cell pellet in four PCV (i.e., 10 mL) of Buffer A, and place on ice for 20 min.
6. Add protease inhibitors: 31 μL of PMSF, 1.25 μL of leupeptin, 6.25 μL of pepstatin, and 6.25 μL of chymostatin.
7. Transfer the cell suspension to an ice-cold Dounce homogenizer and lyse the cells by homogenization with 15 strokes using a B pestle (see Note 2).
8. Add four PCV (i.e., 10 mL) of ice-cold buffer B and mix slowly using a Pasteur pipet while stirring.
9. Transfer the homogenate to a precooled 100-mL glass beaker, and slowly add one PCV (i.e., 2.5 mL) of saturated $(\text{NH}_4)_2\text{SO}_4$ dropwise with gentle mixing.

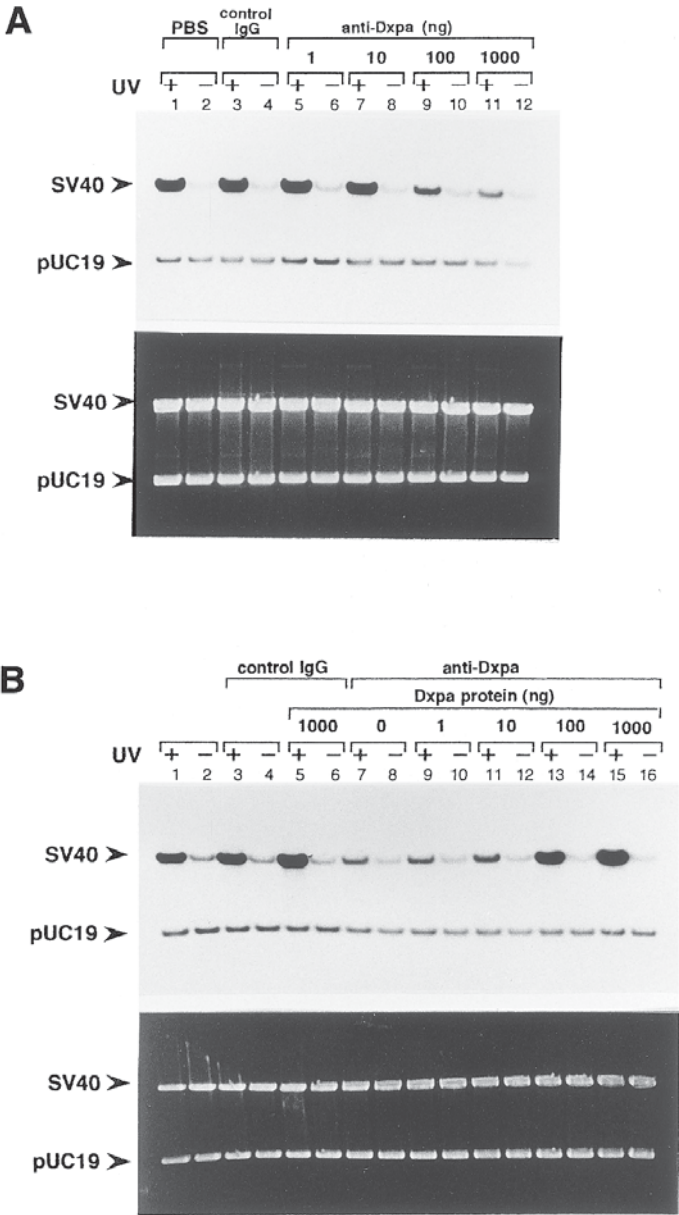


Fig. 1. NER in Kc whole-cell extracts. (A) Nucleotide excision repair synthesis and its inhibition by affinity-purified anti-Dxpa antibodies. UV-irradiated (lanes 1, 3, 5, 7, 9, and 11) or unirradiated (lanes 2, 4, 6, 8, 10, and 12) SV40 minichromosomes were incubated in standard reaction mixtures, which were preincubated in the presence of 1 μ g of control rabbit IgG (lanes 3 and 4) or 1 ng (lanes 5 and 6), 10 ng (lanes 7 and 8),

10. Stir the viscous lysate very gently (to prevent DNA shearing) for 30 min on ice (*see Note 3*).
11. Transfer the extract very carefully into 5-mL polyallomer ultracentrifuge tubes.
12. Centrifuge the extract at 45,000 rpm for 3 h at 2°C in a SW 50.1 rotor (Beckman) (243,000*g*).
13. Decant the supernatant so as to not disrupt the pellet (the last 1 or 2 mL are left behind) and transfer (~16 mL) to an ice-cold beaker.
14. Precipitate proteins by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ (0.33 g/mL of solution; i.e., 5.28g).
15. After the $(\text{NH}_4)_2\text{SO}_4$ is dissolved, add 1 *N* NaOH (0.1 mL/10 g solid $(\text{NH}_4)_2\text{SO}_4$) (i.e., 53 μL of 1 *N* NaOH), and stir the suspension for 30 min.
16. Centrifuge the suspension at 15,000 rpm for 20 min at 2°C in SW 50.1 rotor (27,000*g*).
17. Discard the supernatant completely.
18. Resuspend the precipitate in a 5% vol of dialysis buffer C (*see Note 4*).
19. Dialyze the suspension in a prewashed dialysis bag against two changes of 100 vol each of ice-cold buffer C for a total of 8–12 h. (The volume of the solution increases 30–50% during dialysis.)
20. Centrifuge the dialyzate at 12,500 rpm (~12,000*g*) for 10 min at 2°C in a microcentrifuge.
21. Collect the supernatant, and measure the protein concentration of the extract (*see Note 5*).
22. Flash-freeze 200- μL aliquots of the extract in 1.5-mL microcentrifuge tubes, and store at –80°C (*see Note 6*).

3.3. In Vitro DNA Repair Assay

1. Prepare a standard reaction mixture of 20 μL per tube as follows:
 - a. 1.6 μL of 0.5 *M* creatine phosphate.
 - b. 0.4 μL of 0.1 *M* ATP.
 - c. 0.3 μL of dNTP-C mixture.
 - d. 0.2 μL of 1 *mM* dCTP.

100 ng (lanes 9 and 10), and 1000 ng (lanes 11 and 12) of anti-Dxpa antibodies for 30 min on ice. Plasmids were linearized and resolved by 1% agarose gel electrophoresis. Upper panel, autoradiogram; Lower panel, ethidium bromide staining of the gel. **(B)** Restoration by recombinant Dxpa protein of the repair synthesis by extract depletion using anti-Dxpa antibodies. One nanogram (lanes 9 and 10), 10 ng (lanes 11 and 12), 100 ng (lanes 13 and 14), or 1000 ng of recombinant Dxpa protein were added to the standard reaction mixtures containing 1 μg of control rabbit IgG (lanes 3–6) or 100 ng of anti-Dxpa antibodies (lanes 7–16) and the DNA repair reaction was performed in the presence of UV-irradiated (lanes 1, 3, 5, 7, 9, 11, 13, and 15) or unirradiated (lanes 2, 4, 6, 8, 10, 12, 14, and 16) SV40 minichromosomes. Upper panel, autoradiogram; lower panel, ethidium bromide staining of the gel.

- e. 0.1 μL of [α - ^{32}P]dCTP.
- f. 0.2 μL of 100X MgCl_2 -DTT solution.
- g. 0.05 μL of 10 mg/mL phosphocreatine kinase.
- h. 1.28 μL of 5 mg/mL BSA.
- i. 5 μL of Kc cell extract (16 $\mu\text{g}/\text{mL}$: total protein 80 μg).
- j. 1 μL of 0.3 mg/mL unirradiated pUC19 RF1 DNA.
- k. 1 μL of 0.3 mg/mL of UV-irradiated or unirradiated SV40 minichromosomes.
- l. Add sterile water up to 20 μL (*see Note 7*).
2. Incubate the samples at 30°C for 3 h.
3. Terminate the reaction by addition of 1 μL of 0.5 M EDTA and 1 μL of 1 mg/mL RNase A on ice, and incubate at 37°C for 30 min.
4. Add 28 μL of Stop solution I.
5. Incubate at 37°C for 1 h.
6. Vortex the mixture vigorously with an equal volume of phenol-chloroform. After centrifugation, carefully remove the aqueous phase, and add 50 μL of 5 M ammonium acetate, 2 μL of 10 mg/mL tRNA as carrier, and 300 μL of ethanol to precipitate the DNA (*see Note 8*). Keep in a -80°C freezer for 30 min.
7. Centrifuge the tubes at top speed ($\sim 15,000g$) for 20 min in a microcentrifuge.
8. Rinse the precipitate in 80% ethanol, and dry the pellet.
9. Dissolve the DNA in 15 μL of 10T1E, and add 5 μL of *Eco*RI reaction mixture.
10. Incubate at 37°C for 2 h.
11. Terminate the reaction by adding of 5 μL of stop solution II.
12. Load the DNA samples onto a 1% agarose gel, and electrophorese at 20–30 V for 12–18 h with TAE buffer.
13. Stain the gel with 1 $\mu\text{g}/\text{mL}$ of ethidium bromide solution, and photograph the stained gel. (*See Note 9*.)
14. Dry the gel under vacuum, and expose to X-ray film with an intensifying screen overnight at -80°C (*see Fig. 1*, shown on p. 342).

4. Notes

1. Kc cells are attached loosely to the culture plate and grow with a doubling time of about 24 h under normal conditions. At confluence, Kc cells number approx $2.5\text{--}5 \times 10^7$ per 150-mm dish.
2. Check the condition of cell lysis with a microscope. Be careful not to create too much frothing during the lysis process to avoid denaturation of proteins.
3. Chill the beaker by placing it in the center of an ice box surrounded by ice. Mix the lysate very slowly using a low-speed, powerful stirrer. Be careful not to shear the DNA, since this may interfere with its removal in the next step.
4. At this scale, we use four ultracentrifuge tubes. Transfer the total volume of buffer C into one tube, and solubilize the precipitate by gently pipeting with a Pasteur pipet so as not to cause frothing. Then transfer the total solution to the next tube, and so on. Although the precipitates cannot be perfectly solubilized, the whole solution and a portion of the insoluble materials are to be transferred to the dialysis bag. Some of the insoluble materials may be solubilized during dialysis.

5. You should obtain about 1 PCV of final whole-cell extract (i.e., 2.2–2.5 mL of whole cell extract prepared from 5×10^8 Kc cells). The protein concentration of the final whole cell extract is 15–20 mg/mL. To prepare a good whole-cell extract, the final protein concentration needs to be >10 mg/mL. This condition is very similar to the preparation of whole-cell extracts employed in the in vitro transcription system described in Manley et al. (14).
6. Whole-cell extracts are stable at -80°C over a year, and can be thawed and quick-frozen several times without loss of activity for NER.
7. Typically we use more than 10 tubes/experiment. Therefore, we prepare more than 10 times the volume of one reaction mixture. Each reaction mixture aliquot contains the following components: 40 mM creatine phosphate, 2 mM ATP, 50 μM dATP, 50 μM dGTP, 50 μM TTP, 10 μM dCTP, 10 μM (1 μCi) [α - ^{32}P]dCTP, 7 mM MgCl_2 , 1 mM DTT, 25 $\mu\text{g/mL}$ phosphocreatine kinase, 320 $\mu\text{g/mL}$ BSA, 4 mg/mL whole-cell extract, 0.3 μg unirradiated pUC19 RF1 DNA, 0.3 μg SV40 UV-irradiated minichromosome. As shown in Fig. 1, to examine the function of proteins in the extract (*Drosophila* homolog of the human XPA gene product: Dxpa), we add either affinity-purified anti-Dxpa antibody or recombinant Dxpa protein or both to the reaction mixture as described in our paper (9).
8. To obtain pure naked SV40 DNA, chromosomal proteins are removed by vortexing vigorously during the phenol-chloroform extraction.
9. Do not apply samples to gels containing ethidium bromide, since this might interfere the running pattern of viral or plasmid DNA. Ethidium bromide staining should be performed only after running the gel.

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Nucleotide Excision Repair in Nuclear Extracts from *Xenopus* Oocytes

Eric J. Ackerman, Lilia K. Koriazova,
Jitendra K. Saxena, and Alexander Y. Spoonde

1. Introduction

Limited nucleotide excision repair (NER) requires at least ~40 proteins in extracts from purified proteins (**1,2**) although perhaps hundreds of proteins may influence DNA repair in cells. For efficient DNA repair in extracts, it is important to utilize a system containing large quantities of active DNA repair proteins uncontaminated with nonspecific nucleases. Unlike extracts derived from mammalian cells that repair ~2% of the input DNA, both injected *Xenopus* oocytes (**3**) and oocyte nuclear extracts can repair ~100% of the input damaged DNA by NER with little or no synthesis on undamaged control substrate (**Fig. 1**). Repair activity in extracts can be inactivated with antibodies and/or inhibitors, and then repair can be restored by addition of exogenous proteins (**4**). A further advantage of the *Xenopus* system is that results obtained from injection experiments in living cells can be compared to results obtained in nuclear extracts.

Fully grown (i.e., stage 6) *Xenopus* oocytes are unusually large (~1-mm diameter) single cells containing a nucleus encompassing ~10% of the nonyolk volume, i.e., each nucleus is ~40 nL. Oocytes are arrested at first meiotic prophase; unlike eggs, oocytes cannot be fertilized. A fertilized egg can replicate DNA faster than *Escherichia coli* in log phase. This rapid replication rate is because nearly 4000 cells' worth of nuclear proteins are stored during oogenesis. These stored DNA polymerases and other accessory proteins support an active DNA repair system that prevents delays in DNA replication on damaged template.

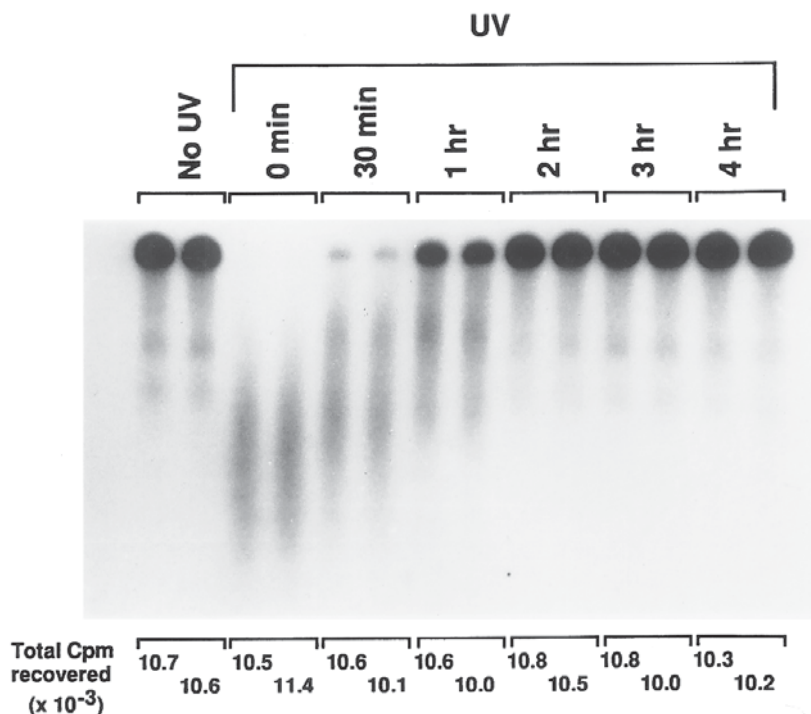


Fig. 1. Rapid repair of cyclobutane pyrimidine dimers. Duplicate 0.2- μ g samples of uniformly labeled UV-irradiated plasmid pSP65 (Promega) containing ~ 4 dimers/plasmid were incubated with nuclear extract containing ~ 2 nuclei/reaction. Recovered DNA was linearized with *Eco*RI, digested with T4 UV endonuclease, electrophoresed on a 1% alkaline-agarose gel, and autoradiographed. Adapted with permission from ref. (4).

This chapter describes preparation of oocyte nuclear extracts based on modifications of earlier methods (5) consisting of:

1. Selecting appropriate oocytes.
2. Separating the fully grown oocytes.
3. Treatment of the oocytes with nonspecific protease to degrade the cell wall partially without releasing the nucleus.
4. Inactivating the protease.
5. Centrifugal release of the nuclei from the cytoplasm.
6. Purification and storage of the nuclei. All materials and methods for using nuclear extracts in DNA repair reactions are also described. Kay and Peng have edited an excellent methods book for generalized *Xenopus* applications (6).

2. Materials

2.1. Preparation of *Xenopus* Oocytes

1. *Xenopus* vendors:

Xenopus I

716 Northside

Ann Arbor, MI 48105

313-426-2083

Burley Lilley Co.

64 S. Jackson St.

Beverly Hills, FL 34465

800-936-6787

fax: 904-527-1417

Nasco

901 Janes Avenue

Fort Atkinson, WI 53538

800-558-9595

414 -563-2446

2. Dissecting microscope.
3. Disposable bacterial Petri dishes.
4. Forceps (Dumont Inox #5).
5. 710 μ m Nitex membrane (50–100 mm diameter) held in a plastic joint as used with Fischer/Porter columns.
6. Refrigerated incubator set at $\sim 16^{\circ}\text{C}$ for storage of Barth's medium and Gurdon's medium (GM) (see **Subheading 2.2.**).
7. Work surface that can be chilled to $\sim 16^{\circ}\text{C}$ with a refrigerated water circulator. This is useful for maintaining oocyte temperature during the selection procedure.

2.2. Preparation of Nuclear Extracts

1. Collagenase and Subtilisin (Sigma, St. Louis, MO, or Worthington, Freehold, NJ).
2. 10X Barth's medium (per 2 L): 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO_3 , 10 mM HEPES, 0.82 mM MgSO_4 , 0.33 mM $\text{Ca}(\text{NO}_3)_2$, and 0.41 mM CaCl_2 .
To make 2 L add: 102.6 g NaCl, 1.5 g KCl, 4.04 g NaHCO_3 , 47.66 g HEPES. Add water to 1.5 L, adjust to pH 7.5, then add in order: 4.04 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.66 mL $\text{Ca}(\text{NO}_3)_2$ and 1.8g $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$.
Adjust the volume to 2 L. Store at 4°C .
3. Gurdon's medium: 88 mM NaCl, 1 mM KCl, 0.33 mM $\text{Ca}(\text{NO}_3)_2$, 0.41 mM CaCl_2 , 2.4 mM NaHCO_3 , 10 mM HEPES, pH 7.6, 0.82 mM MgCl_2 .
To make 2 L add: 176 mL 1 M NaCl, 2 mL 1 M KCl, 0.66 mL 1 M $\text{Ca}(\text{NO}_3)_2$, 0.82 mL 1 M CaCl_2 , 4.8 mL 1 M NaHCO_3 , 20 mL 1 M HEPES, pH 7.6, 1.64 mL 1 M MgCl_2 and H_2O to 2 L. Store at 16°C . (See **Note 1.**)
4. GM containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) (GM-PMSF): Add 2 mL of 0.25 M PMSF (0.44 g/10 mL ethanol) to 1000 mL of GM.
The PMSF should be added just before use. Store at 4°C .

5. SMT-1 Medium: 0.25 *M* sucrose, 1 *mM* MgCl₂, 10 *mM* Tris-HCl, pH 7.4, 0.2% Triton X-100, 2.5 *mM* dithiothreitol (DTT), 0.2 *mM* PMSF.
To make 2 L add: 171.12 g sucrose, 2.0 mL 1 *M* MgCl₂, 20 mL 1 *M* Tris-HCl, pH 7.4, 4.0 mL Triton X-100, 5.0 mL DTT and 1.6 mL 0.25 *M* PMSF. Add the DTT, and PMSF just before use. Store at 4°C.
6. SMT-2 medium: 0.25 *M* sucrose, 3 *mM* MgCl₂, 10 *mM* Tris-HCl, pH 7.4, 0.05% Triton X-100, 2.5 *mM* DTT, 0.2 *mM* PMSF.
To make 2 L add: 171.12 g sucrose, 6.0 mL 1 *M* MgCl₂, 20 mL 1 *M* Tris-HCl, pH 7.4, 1.0 mL Triton X-100, 5.0 mL 1.0 *M* DTT, and 1.6 mL PMSF. Add the DTT and PMSF just before use. Store at 4°C.
7. J-buffer: 70 *mM* KCl, 7 *mM* MgCl₂, 0.1 *mM* EDTA, 2.5 *mM* DTT, 10% v/v glycerol, 10 *mM* HEPES, pH 7.4, 1% polyvinylpyrrolidone (PVP) 360.
To make 1 L add: 70 mL 1.0 *M* KCl, 7.0 mL 1 *M* MgCl₂, 0.2 mL 0.5 *M* EDTA, 2.5 mL 1.0 *M* DTT, 100 mL glycerol, 10 mL 1.0 *M* HEPES, pH 7.4, and 10 g PVP. Add the DTT just before use. Store at 4°C.

2.3. DNA Repair Reaction

1. Protein concentration determination kit (Pierce, Rockford, IL).
2. 3X J-buffer: Store in aliquots at -70°C. DTT deteriorates after 3 mo.
3. 10X Chase buffer: 0.5 *mM* each of dATP, dGTP, dCTP, TTP (Pharmacia, Piscataway, NJ). Store in aliquots at -70°C. Replace about every 4 mo.
4. α-[³²P]dCTP. Use 2.5 μCi α-[³²P]dCTP in label incorporation assays such that total CTP concentration is 50 μM in the reaction.
5. Proteinase K (60 mg/mL) stock solution. Store in aliquots at -70°C.
6. Stop solution: 10 *mM* Tris-HCl, pH 7.5, 15 *mM* EDTA, 0.3% SDS. Immediately before use, add 16 μL of freshly-thawed 60 mg/mL proteinase K to 10 mL stop solution, i.e., final stop solution contains 96 μg/mL proteinase K. Use this solution for only one experiment, and then discard. Do not store stop solution containing proteinase K!

2.4. UV-Endo Reaction

1. 10X UV endo buffer: 1.5 *M* NaCl, 0.1 *M* Tris-HCl, pH 8.0, 0.15 *M* EDTA.
2. TE: 10 *mM*, Tris-HCl, pH 7.5, 0.1 *mM* EDTA.
3. T4 UV endonuclease (gift of R. S. Lloyd, University of Texas, Galveston).

2.5. DNA Replication Reaction

1. Single-stranded M13 phage DNA (New England Biolabs, Beverly, MA).
2. 10X ATP regenerating system: 200 *mM* Tris-HCl, pH 7.5, 125 *mM* MgCl₂, 4 *mM* DTT, 60 *mM* phosphocreatine (Sigma), 100 U/mL creatine phosphokinase (Sigma), 10 *mM* rATP (Pharmacia). Store in aliquots at -70°C.

2.6. Alkaline-Agarose Gel Electrophoresis

1. Electrophoresis-grade agarose.
2. Alkaline-agarose gel buffer: 50 *mM* NaCl, 1 *mM* EDTA.

3. Electrophoresis running buffer (prepared fresh): 30 mM NaOH, 1 mM EDTA.
4. Loading buffer: 50 mM NaOH, 1 mM EDTA, 2.5% Ficoll, 0.025% bromophenol blue. Make up 100 mL of 1 mM EDTA, 2.5% Ficoll 400, and 0.025% bromophenol blue. To 0.5 mL of this solution, add 2.5 μ L 10 N NaOH. The 10 N NaOH solution should not be more than 8 wk old. The final loading buffer therefore contains 50 mM NaOH.
5. 7.5% Trichloroacetic acid (TCA) solution.
6. Schleicher & Schuell (S&S) gel blotting paper.
7. Whatman 3MM paper.

2.7. Preparation of Uniformly Labeled DNA

Minimal phosphate medium: of 20 mM KCl, 100 mM NaCl, 20 mM NH_4Cl , 1 mM CaCl_2 , 100 mM Tris-HCl, pH 7.4, 0.1% Casamino acid, and 0.1% Bacto-peptone. Filter separately and add to the above autoclaved solution: glucose to 0.2%, fructose to 0.2%, MgSO_4 to 0.2 mM, 0.3 mM threonine to 0.3 mM, leucine to 0.3 mM, and thiamine to 0.00005%.

3. Methods

3.1. Isolation of *Xenopus* Nuclei

Oocytes from four frogs require ~1 L GM, 2 L GM-PMSF, 1 L SMT-1, 1 L SMT-2, and 500 mL of J buffer.

1. Separate ovaries into groups of 30–50 oocytes/group, and place into a 600-mL beaker containing 1X Barth's buffer (sufficient to cover them) at 25°C (*see Note 2*).
2. Add 100 mL/ovary of Barth's buffer containing 0.25% collagenase, and incubate at 25°C for 4–5 h with occasional stirring (~1 min of swirling every 15 min or, alternatively, gentle rocking on a rocker platform) until nearly all clumps have been separated into single oocytes.
3. Wash the oocytes with gentle stirring in 1000 mL of Barth's buffer (5 $\times$ 200 mL washes).
4. Separate the mature oocytes from debris and smaller oocytes by filtering through a 710- μ m Nitex membrane held in a plastic joint as used with Fischer/Porter columns (this retains stage VI oocytes, allowing stage V and IV to pass through).
5. Wash the oocytes in GM and leave at 20°C in a beaker, covered with the same medium. (*See Note 3.*)
6. Gently pipet a sufficient number of oocytes to cover the bottoms of two flat-bottom 250-mL centrifuge bottles completely. Add 15 mL of GM containing 0.15% subtilisin, and incubate at 20°C for 3 min. (*See Note 4.*)
7. Immediately add 30 mL of cold GM-PMSF (to inhibit the subtilisin). The centrifuge bottles should be kept on ice during this and subsequent steps.
8. Remove the supernatant by suction and wash the oocytes twice with 30 mL of cold GM-PMSF.

9. Wash the oocytes with 20 mL of cold SMT-1.
10. Add 20 mL of SMT-1, and incubate on ice for 15 min.
11. Centrifuge at 200g for 5 min at 4°C.
12. Add 40 mL of cold SMT-2 to stabilize the released nuclei.
13. Stir the centrifuge tubes gently in a rotary fashion to resuspend the nuclei while leaving the oocyte membranes and yolk undisturbed.
14. Quickly filter the supernatants (nuclei are present in the last few milliliters) through a 40-mm Nitex membrane, which is held in a Fischer/Porter joint and supported in a crystallization dish containing cold SMT-2 to a height of several centimeters. Rapidly transfer the 40-mm Nitex membrane to a fresh dish containing cold J buffer, and gently wash away the yolk and debris.
15. Quickly collect nuclei from the membrane using a glass pipet and place into a Petri dish containing cold J buffer.
16. Rapidly collect ~30–50 nuclei/tube in ~20–60 μ L of J buffer, using a 200- μ L pipet with the tip cut near the end so that nuclei may enter/exit without breaking (select only round/intact nuclei uncontaminated with yolk). Immediately freeze the nuclei in dry ice/ethanol and store at -80°C . (See **Note 5**.)

3.2. DNA Repair Reaction (see Note 6)

1. Thaw nuclei prepared in **Subheading 3.1.** on wet ice. Do not thaw at 37°C . Do not thaw nuclei until after everything else necessary for the reaction is ready.

Each reaction uses 9 μ L of extract at ~4 nuclei/9 μ L. Dilute each -80°C aliquot appropriately with ice-cold J buffer after the nuclei have thawed on wet ice. Because precise estimation of the number of nuclei per tube is difficult, protein concentration can be used as an alternative. Generally, efficient nuclear extract should have a protein concentration not <0.5 mg/mL.

2. Measure the volume of the nuclear aliquot after thawing on wet ice.
3. Spin out the debris at 14,000g for ~3 s in a refrigerated microcentrifuge.
4. Quickly return the tube to ice.
5. Determine the protein concentration.
6. Add fresh DTT to 3X J buffer (7.5 μ L to 1 mL of 3X J buffer).
7. Set up the repair reaction components (see **Note 7**):

10X Chase buffer (see Note 8)	3 μ L
2.5 μ Ci α -[^{32}P]dCTP (label incorporation assay)	
Damaged DNA (0.2 mg/mL) (see Note 9)	2 μ L
3X J-Buffer	10 μ L
H ₂ O	2 μ L
8. Flick/spin twice (do not vortex), then add 5 μ L of freshly thawed extract from **step 3**, and bring the reaction volume to 30 μ L with H₂O. Flick/spin (do not vortex!).
9. Incubate at room temperature in the dark for up to 4–6 h.
10. Add 30 μ L of stop solution, and then incubate for 30 min at 37°C .
11. Add 150 μ L tRNA carrier (0.5 mg/mL). Add 200 μ L of phenol:chloroform. Vortex and then centrifuge for 15 min at room temperature.

12. Transfer the aqueous phase to a fresh Eppendorf tube, and precipitate the DNA by adding 20 μL of 3 M NaOAc, 700 μL of ethanol, and vortexing. Put the tube on dry ice for 15 min. Spin at 14,000g for 20 min at 4°C.
13. Wash the pellet with 100 μL of cold 70% ethanol. Spin at 4°C for 10 min. Remove the ethanol, and air-dry the pellet.
14. Linearize the DNA sample with an appropriate restriction enzyme for easier gel analysis.

3.3. UV-Endonuclease Reaction

1. To the dried pellet from the DNA repair reaction add: 18 μL of TE and 2 μL of 10X UV-Endo buffer. Vortex and spin twice.
2. Remove 9 μL of sample for UV-endonuclease treatment.
3. Add 1 μL of UV-endonuclease to 9 μL reaction. Flick/spin. Incubate at 37°C for 45 min.
4. Transfer 5 μL of the reaction and 5 μL of a “minus UV-endonuclease” control to fresh tubes. Dry the samples in a SpeedVac. The samples are now ready for alkaline-agarose gels.

3.4. Alkaline-Agarose Gel Electrophoresis and Gel Processing

1. Reflux 1 g of agarose with 100 mL of alkaline agarose-gel buffer. After the gel solidifies, equilibrate for 1 h to overnight in alkaline-agarose gel running buffer.
2. Dissolve the samples in 10 μL of loading buffer. Vortex and spin before loading the gel.
3. Electrophorese overnight at 30 V.
4. For each gel, prepare 1 L of 7.5% TCA.
5. Place the gel in a glass tray containing 500 mL of 7.5% TCA.
6. After 15 min, remove the old TCA and add the remaining 500 mL of fresh TCA. Leave at room temperature for 15 min.
7. Dry the TCA-soaked gel by laying it on top of a stack of S&S blotting paper with a single piece of Whatman 3MM on top of the stack. Place Saran wrap and a Plexiglas sheet on top of the gel. Place a 1-L bottle on top of the Plexiglas.
8. When the gel is dry (after ~2 h), expose it to X-ray film.

3.5. ^{32}P -Labeling Protocol for Uniformly Labeled Plasmid (10^6 dpm/mg) (see Note 10)

1. Inoculate a single bacterial colony in rich medium (e.g., Superbroth) containing the appropriate antibiotic.
2. Grow overnight at 37°C with shaking.
3. Add 0.5 mL of starter culture to 30 mL of minimal phosphate medium in a 250-mL disposable Erlenmeyer flask. Grow for 45 min in a shaker/incubator.
4. Add ~5–10 mCi [^{32}P] orthophosphoric acid (8500 Ci/mmol).
5. Place the flask inside a QuickSeal™ bag in case of spills.
6. Grow for 5 h and then isolate form I plasmid by CsCl ultracentrifugation (3).

4. Notes

1. There is very little difference between Barth's medium and GM. However, we have never simplified our procedures to only one medium.
2. Oocytes enter heat shock at 29°C. Be careful when warming cold solutions in a 37°C water bath to bring them to room temperature first.
3. Because the nuclei lyse quickly after they have been isolated, it is best to prepare them in several smaller batches beginning at **step 5**.
4. The time for optimal subtilisin treatment must be determined empirically based on the initial preparations. The yield of nuclei is best when ~30% of the oocytes burst during incubation in Triton X-100.
5. Work quickly when separating nuclei. Freezing in liquid nitrogen rather than dry ice:ethanol does not appear to improve activity. Extracts are stable for years when stored at -70°C, and once thawed are active for at least 8 h. In some cases, extracts can be refrozen with no loss of activity.
6. Single-strand (ss) to double-strand (ds) replication reactions (**7**) are a more convenient assay than NER to test the efficiency of the extracts. In our hands, we have never encountered a batch of extract that was active in repair, but inactive in ss to ds replication. Therefore, initial attempts at making extracts can be easily evaluated by testing for replication activity. It is necessary to include an ATP-regenerating system for the replication reaction, but we recently found this ATP-regenerating system is unnecessary for DNA repair reactions.
7. For the replication reaction, the buffer should include the ATP-regenerating system and rNTP solution (*see Subheading 2.5.*).
8. dCTP in the chase buffer must be lowered or eliminated if using α -[³²P]dCTP in label incorporation assays.
9. Damaged DNA can be prepared by UV irradiation or chemical treatment (**4**). pUC18 DNA containing ~5 AAF adducts/plasmid is available commercially from Texagen (Plano, TX).
10. The procedure for ³²P-uniformly labeled plasmid is used only when it is necessary to monitor conversion of damaged plasmid to repaired plasmid without resorting to label incorporation.

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Assay for Nucleotide Excision Repair Protein Activity Using Fractionated Cell Extracts and UV-Damaged Plasmid DNA

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1. Introduction

Mammalian cells remove carcinogenic damage caused to DNA by ultraviolet (UV) light and certain other mutagens mainly by using the pathway known as nucleotide excision repair (NER). This involves damage recognition, unwinding of the DNA around the site of damage, incision on either side of the lesion, removal of a fragment containing the lesion, and finally DNA synthesis and ligation to form a repair patch of ~30 nucleotides. The use of purified proteins and reconstituted systems has revealed the protein components that are essential for the core dual-incision reaction (**1**). XPA and the single-strand binding protein replication protein A (RPA) associate with each other and preferentially bind to damaged DNA. XPC, which usually exists bound to its partner protein hHR23B, is also involved in damage recognition. TFIIH, a transcription initiation complex, which includes the XPB and XPD helicases as subunits (**2**), is involved in local opening of DNA around the site of damage (**3**). The incisions are made by two structure-specific endonucleases, XPG and the ERCC1-XPF complex (**4–6**). These proteins cleave the damaged strand of the unwound DNA 3' and 5' to the lesion respectively. A fragment of ~24–32 nucleotides containing the lesion is released and the gap is filled by a DNA repair synthesis reaction involving the proliferating cell nuclear antigen- (PCNA) dependent DNA polymerase δ or ϵ holoenzyme (**7**) using the undamaged strand as a template, and the patch is joined by a DNA ligase.

Repair synthesis can be measured by incubating damaged circular plasmid DNA with cell-free extracts in a reaction mixture that includes radiolabeled

deoxynucleoside triphosphates. The incorporation can be quantified and used as a measure of the amount of repair (8,9). This assay is useful for measuring the activity of individual purified DNA repair proteins (6) and to observe in vitro complementation between different repair-defective cell extracts (10). The reaction can also be resolved into incision and polymerization stages by using fractionated cell extracts (11) or reconstituted by mixing fractionated extracts back together. Other reagents, such as antibodies, enzymes, and inhibitors, can be added to the cell-free system. DNA incorporating different lesions or treated with various types of chemicals or radiation, or containing a single lesion at a defined site can be used. Repair synthesis has been used as an assay with DNA assembled into nucleosomes (12–14).

The system has been adapted to measure repair synthesis in extracts from other organisms, including the budding yeast *Saccharomyces cerevisiae* (15,16; see Chapter 25), the fission yeast *Schizosaccharomyces pombe* (see Chapter 26), *Xenopus* (14,17), and *Drosophila* (18, see Chapter 27). Extracts from fresh human malignant lymphoid cells have also been used (19). Other sources of cells, such as tissue samples, may present difficulties caused by the diversity of cell types and cell-cycle states within the tissue sample, and can contain regions of dying cells that can be sources of degradative enzymes.

Although the methods are straightforward, it is a considerable amount of work to set up the cell-free repair synthesis assay in a laboratory. Major investments of time and effort are required to grow sufficient quantities of healthy cells, maintain stocks of active extracts, and prepare a large amount of highly purified DNA substrate with low background.

For the purpose of assaying the activity of an individual repair protein, it is most straightforward to use a cell-free extract from a mutant cell line lacking the corresponding activity. The extract is fractionated on a phosphocellulose column (11,20) using conditions that separate PCNA and RPA from the rest of the repair proteins (CFII). Purified RPA is added back at the incision stage and purified PCNA for a short pulse at the synthesis stage during the assay. There should be no repair synthesis in the damaged DNA using the mutant CFII and RPA alone and a clear specific complementation when protein is added. Very little or no signal occurs in the undamaged DNA. The use of CFII to assay for purified proteins results in much lower background repair synthesis than unfractionated extract. The DNA substrate is damaged plasmid and does not need to contain a single specific lesion, although it should be carefully prepared and in the case of UV-damaged DNA much better results are achieved by removing pyrimidine hydrate photoproducts from the substrate using *Escherichia coli* Nth protein. Other, more detailed questions relating to the incision stage of NER can be answered in vitro using dual-incision assays, such as those described in Chapter 30.

2. Materials

2.1. Plasmid Preparation

1. Luria broth (LB).
2. LB agar plates.
3. Ampicillin.
4. Qiagen DNA purification kit (Qiagen).
5. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
6. Petri dishes (Sterilin).
7. Germicidal lamp (254 nm peak wavelength).
8. 10X Nth buffer: 0.4 M HEPES-KOH, pH 8.0, 1.0 M KCl, 5.0 mM EDTA, 5.0 mM dithiothreitol (DTT), and 2 mg/mL bovine serum albumin (BSA) (nuclease-free; Gibco BRL)
9. Nth protein (endonuclease III). (*See Note 1.*)
10. Cesium chloride (Gibco BRL).
11. Ethidium bromide: 10 mg/mL (Sigma).
12. Syringes and needles.
13. Aluminum foil.
14. Quick-Seal centrifuge tubes (Beckman).
15. Ti60 rotor.
16. CsCl/isopropanol/TE: Dissolve 10 g of CsCl in 10 mL of TE, and then add 50 mL of isopropanol (an extra 1–2 mL of TE may be needed to allow the CsCl to go into solution).
17. Absolute ethanol.
18. Thick-walled 25 × 89 mm centrifuge tubes (Beckman).
19. SW28 ultracentrifuge rotor (Beckman).
20. Sucrose, UltraPure (Gibco BRL).
21. Sucrose gradient buffer: 25 mM Tris-HCl, pH 7.5, 1 M NaCl, 5 mM EDTA.
22. Peristaltic pump.
23. Magnetic stirrer.
24. Ultraclear centrifuge tubes 25 × 89 mm (Beckman).
25. Agarose (molecular biology grade).
26. 70% Ethanol.

2.2. Cell Extract Preparation

1. 175-cm² tissue-culture flasks (Falcon).
2. 850-cm² roller bottles (Falcon No. 3027).
3. Phosphate-buffered saline A (PBSA): 10 g/L NaCl, 0.25 g/L KCl, 0.25 g/L KH₂PO₄, 1.43 g/L Na₂HPO₄.
4. 0.25% Trypsin (Gibco BRL) and phosphate-buffered saline.
5. Fetal calf serum (FCS) (Gibco BRL).
6. Hypotonic lysis buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM DTT.
7. Phenylmethylsulfonyl fluoride (PMSF): 500 mM in dry methanol (Sigma) or 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF): 100 mM in H₂O (Calbiochem).

8. Leupeptin: 5 mg/mL in H₂O (Sigma).
9. Pepstatin: 5 mg/mL in dimethyl sulfoxide (DMSO) (Sigma).
10. Chymostatin: 5 mg/mL in DMSO (Sigma).
11. Aprotinin (Sigma).
12. Glass homogenizer with Teflon pestle.
13. Sucrose glycerol buffer: 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 2 mM DTT, 25% sucrose (Gibco BRL), 50% glycerol (Fluka).
14. Ammonium sulfate (BDH Analar).
15. Cell extract dialysis buffer: 25 mM HEPES-KOH, 0.1 M KCl, 12 mM MgCl₂, 1 mM EDTA, 17% glycerol (Fluka), 2 mM DTT (add just before use), pH to 7.9 with 5 M KOH.
16. Phosphocellulose P11 (Whatman).
17. Buffer A: 25 mM HEPES-KOH, pH 8.0, 10% glycerol, 150 mM KCl, 1 mM EDTA, 2 mM DTT.
18. Chromatography column (1.6 cm diameter) to hold 5 mL of resin.
19. Amicon stirred pressure cell (Amicon).

2.3. Repair Reactions

1. 5X buffer for repair reactions: 200 mM HEPES, 25 mM MgCl₂, 2.5 mM DTT (Sigma), 10 mM ATP (Sigma) pH 5.2, 100 μ M dGTP, 100 μ M dCTP, 100 μ M TTP, 40 μ M dATP (nucleotide triphosphates from Pharmacia, 100 mM), 110 mM phosphocreatine (Sigma di-Tris salt), 1.7 mg/mL BSA (nuclease-free, Gibco BRL), adjust the pH with 1 M KOH to give pH 7.8 in the final reaction (set up a mock reaction to test this).
2. Creatine phosphokinase (CPK, rabbit muscle; Sigma), 2.5 mg/mL in 10 mM glycine, pH 9.0, and 50% glycerol. Store at -20°C .
3. [α -³²P] dATP (3000 Ci/ mmol; Amersham).
4. 10% Sodium dodecyl sulfate (high quality, e.g., BDH Analar).
5. Proteinase K: 2 mg/mL (Sigma).
6. RNase A: 2 mg/mL (Sigma).
7. Phenol/chloroform/isoamyl alcohol (25:24:1) (Fluka).
8. 0.5 M EDTA, pH 8.0.
9. 7.5 M Ammonium acetate.
10. Absolute ethanol (-20°C).
11. RPA (*see Note 2*).
12. Whole-cell extract or CFII.
13. PCNA (*see Note 3*).
14. DNA mix: UV-damaged plasmid and a different-sized undamaged plasmid (control) (*see Note 4*).
15. BamHI restriction endonuclease (Boehringer Mannheim).
16. Kodak film and cassette with screens or phosphorimager cassette and phosphorimager.

3. Methods

3.1. Preparation of DNA Substrate

3.1.1. Plasmid Purification

1. Make fresh transformants of each plasmid to be used in the assay in a strain of *E. coli* containing the *recA* and *endA* mutations (see **Note 4**). Grow overnight at 37°C in LB containing 25 µg/mL ampicillin.
2. Spread 100 µL of transformed cells on a freshly prepared LB agar plate containing 25 µg/mL of ampicillin, and incubate overnight at 37°C.
3. Inoculate one bacterial colony into 10 mL of LB + ampicillin. Incubate overnight at 37°C with shaking.
4. Transfer 2 mL of this culture into each of five 2-L flasks containing 500 mL of LB + ampicillin. Incubate overnight at 37°C with shaking.
5. Prepare plasmid by the Qiagen method using the appropriate-sized pack following the manufacturer's instructions (see **Note 5**).

3.1.2. UV Irradiation

1. Dilute the plasmid to 50 µg/mL in TE.
2. Small volumes of plasmid can be irradiated in 20–50 µL drops in a Petri dish. Pour larger amounts 15 mL at a time into 15-cm Petri dishes, and place on a rocker.
3. Remove the lid of the Petri dish, and irradiate at a dose rate of 0.5 J/m²/s with UV light (peak wavelength of 254 nm), while the dish is rocked to give an even fluence over the surface for 15 min. This gives a total of 450 J/m² (see **Note 6**).
4. Collect the irradiated plasmid and measure the volume.

3.1.3. Treatment with Nth Protein

1. Add 1/9 vol of 10X Nth protein buffer. Keep a 250-ng sample for gel electrophoresis.
2. Add the appropriate amount of Nth protein as calculated by titration (see **Note 1**).
3. Incubate at 37°C for 30 min followed by inactivation at 65°C for 2 min. Keep a 250-ng sample for gel electrophoresis.
4. Mix the 250-ng samples reserved from the unirradiated and irradiated plasmids, before and after Nth protein treatment and measure the amount of nicking by gel electrophoresis (see **Note 1**).

3.1.4. Cesium Chloride Equilibrium Centrifugation

1. Add 1.1 g of cesium chloride powder to the plasmid solution, and mix until fully dissolved. Carefully transfer 30-mL aliquots into 33 mL Beckman Quick-Seal tubes using a syringe with some tubing on the end. Wrap the tubes in aluminum foil, and add 3 mL of ethidium bromide at 10 mg/mL into the top using a syringe and needle. Seal the tubes according to the manufacturer's instructions (see **Note 7**).
2. Mix thoroughly by inverting the tubes, and centrifuge at 38,000 rpm in a Ti 60 rotor (145,000g) for 60 h at 18°C.

3. Remove the band of closed-circular plasmid using a syringe and needle. Work in the dark room under a safe light, and visualize the bands by brief illumination using a 365-nm lamp.
4. Recover the DNA by shaking in an equal volume of CsCl/isopropanol/TE solution in the dark under a safe light. Remove the top, pink layer.
5. Repeat the extraction two to three times until the solution is colorless.
6. Measure the volume and add 3 vol of TE to each, and then 2 total volumes of ethanol.
7. Divide between several thick-walled 25 × 89 mm centrifuge tubes. Mix and leave at −20°C for at least 30 min.
8. Centrifuge at 20,000 rpm (72,000g) for at least 20 min in an SW28 rotor. Carefully wash the pellet in 70% ethanol and resuspend in TE.

3.1.5. Sucrose Density Centrifugation

1. Make up fresh solutions of 5 and 20% sucrose in sucrose gradient buffer.
2. Pour one gradient/500 µg of plasmid DNA. Place 40 mL of the 5% solution in a 100-mL beaker containing a stir bar, on a magnetic stirrer. Pour 40 mL of the 20% solution into another beaker, and pump the 20% solution into the beaker containing the 5% solution (stirring the 5% solution constantly) and at the same time pump two lines from the 5% solution into the bottom of two 25 × 89 mm ultraclear centrifuge tubes. Use a peristaltic pump set to deliver 3–4 mL/min (*see Note 8*). Leave at 4°C for at least 1 h.
3. Precool a rotor and buckets to 4°C. Carefully layer 1 mL of DNA (500 µg) in TE buffer onto the top of each gradient (*see Note 9*). Centrifuge at 25,000 rpm (113,000g) at 2°C in an SW28 rotor for 19 h.
4. Carefully remove the tubes from the buckets, and collect 1.5-mL fractions by pumping out from the bottom. Keep the gradients cool.
5. Load 10 µL of each fraction onto a 0.8% agarose gel containing 0.25% ethidium bromide (*see Note 10*). Place loading buffer in the end wells as a marker. Separate by electrophoresis at 100 V for 3–4 h.
6. The closed-circular DNA should be present in about four to five fractions in the lower third of the gradient (**Fig. 1**). Pool the fractions containing the closed circular DNA and no dimerized or nicked forms, and add 2 vol of ethanol, chill for 10 min, and centrifuge at 20,000 rpm (72,000g) in an SW28 rotor. Rinse with 70% ethanol, centrifuge again, dry the pellet, and resuspend in 1 mL of TE buffer/gradient.

3.1.6. Preparation of DNA Mixture

Check that each plasmid has an acceptably low level of nicked DNA. Examine 250 ng of each one separately by electrophoresis on a 0.8% agarose gel containing 0.25% ethidium bromide. Prepare a “DNA mix” by combining equal amounts of purified damaged and undamaged plasmid. For repair reactions the DNA concentration should be 50 µg/mL of each plasmid in TE (before combining). This is equivalent to 250 ng of each plasmid in a 10-µL aliquot. Check

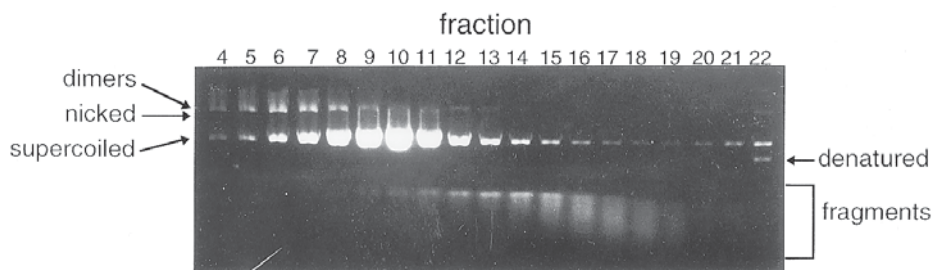


Fig. 1. Sucrose density gradient centrifugation of UV-damaged plasmid DNA. pBluescript KS+ plasmid DNA was isolated on a Qiagen giga column, UV-irradiated, Nth-treated and the supercoiled form isolated after cesium chloride equilibrium centrifugation. Five hundred micrograms of this were separated on a sucrose gradient; 1.6-mL fractions were collected, and 10 μ L of each subjected to electrophoresis on a 0.8% agarose gel containing 0.25 μ g/mL ethidium bromide. Arrows indicate the form of DNA: Supercoiled plasmid DNA is the major species. Dimeric plasmid is present in fractions 4–8. Nicked forms migrate slightly faster than dimers and are mainly removed by the cesium chloride equilibrium centrifugation, but a small amount remains in fractions 11 and 12. Fragments of DNA can be seen in fractions 10–18 and it is important to remove as much of this material as possible from the preparation, since it inhibits repair synthesis (21). Denatured forms occur occasionally as a result of the alkaline lysis step. Fractions 9, 10, and 11 were pooled and reprecipitated for use in the final DNA mix.

the activity of the mixture in a repair assay, using a repair-proficient cell extract. Store the DNA mix in aliquots at -80°C .

3.2. Preparation of Fractionated Cell Extracts

3.2.1. Growth and Harvesting of Fibroblasts

1. Grow fibroblasts in 175-cm² tissue-culture flasks or 850-cm² roller bottles turning at 10 rpm. Fractionated extracts will require about 12 roller bottles or 10^9 cells (see Note 11).
2. When the cells are just confluent, wash twice with 100 mL/bottle of sterile PBSA.
3. Decant the PBSA, and add 100 mL 0.25% Trypsin/PBSA until the cells round up and are almost ready to detach. Decant the trypsin solution.
4. Add 50 mL of medium + 15% fetal calf serum (FCS), and gently agitate until the cells detach. Centrifuge for 5 min at 1000 rpm in a Sorvall HB-4 rotor (165g) to pellet the cells gently.
5. Gently resuspend in 50 mL of medium + 15% FCS and pellet again.
6. Resuspend in 25 mL of cold PBSA (4°C), and pellet again.
7. Resuspend in 25 mL of cold PBSA, and transfer to a 30-mL Corex tube. Centrifuge at 1500 rpm (370g) for 10 min at 4°C . Carefully remove the supernatant, and measure the packed cell volume (PCV).

3.2.2. Growth and Harvesting of Lymphoblastoid Cells

1. Start with 1–2 L of actively growing cells in suspension at $6\text{--}8 \times 10^5$ cells/mL (see **Note 11**). For a fractionated extract, use 10^8 L or 10^9 cells.
2. Centrifuge at 1200 rpm (HB-4 rotor; 235g) for 10 min to pellet the cells and resuspend in cold PBSA. Combine the pellets and wash twice more.
3. After the final wash, carefully remove the supernatant, and measure the PCV.

3.2.3. Whole-Cell Extracts (see **Note 12**)

1. Resuspend the cells in 4 PCV of hypotonic lysis buffer and leave on ice for 20 min. Add per 1 mL of PCV: 5 μ L of 87 mg/mL PMSF or 0.1 mM AEBSF (final concentration); 2 μ L each of 5 mg/mL leupeptin, pepstatin, and chymostatin; and 50 μ L of aprotinin.
2. Break the cells in a glass homogenizer with a Teflon pestle (about 20 strokes). Check by dye exclusion that the cells are broken (see **Note 13**).
3. Transfer the homogenate to a glass beaker in a tray of ice over a magnetic stirrer in the cold, and stir very slowly.
4. Gently add 4 PCV of sucrose-glycerol buffer, and mix carefully.
5. Slowly add 1 PCV of saturated ammonium sulfate solution (see **Note 14**).
6. Pour carefully into polyallomer tubes for an SW55 rotor (or SW50). Do not pipet (see **Note 15**).
7. Centrifuge for 3 h in an SW50 or SW55 rotor at 42,000 rpm (213,000g), at 2°C.
8. Carefully remove the supernatant with a Pasteur pipet, leaving the last 1 mL or 1 cm above the pellet in the tube (see **Note 16**). Measure the volume of supernatant (usually 6–7 mL/mL of PCV). Transfer to a 35 mL polyallomer tube on ice, with a magnetic stirring bar in the bottom of the tube.
9. Slowly add 0.33 g/mL of finely powdered solid ammonium sulfate with mixing. When it is dissolved, add 10 μ L of 1 M NaOH/g of ammonium sulfate added. Stir for 30 min more.
10. Centrifuge for 30 min (for a CFII) or 1 h (for a nonfractionated extract) at 11,000 rpm (~20,000g) in an HB-4 rotor at 4°C.
11. Remove the supernatant, leaving the pellet as dry as possible. Resuspend the pellet in just enough dialysis buffer to allow it to be drawn up into a 1-mL syringe (without the needle) or a cut-off 1 mL pipet tip. For a whole-cell extract, do not dilute in dialysis buffer, since this will yield an extract with very low protein concentration. The suspension should be very thick and milky.
12. Transfer the suspension into a prepared dialysis bag, and clamp it tightly, making sure there are no bubbles trapped inside the bag. Dialyze 1–2 h in 500 mL of extract dialysis buffer. For a CFII, dialyze into extract dialysis buffer containing 150 mM KCl. Dialyze in the cold, and then change the buffer and continue dialysis for 8–12 h in 2 L of fresh buffer. Alternatively, change the buffer three times, and dialyze for only 6 h.
13. Remove the dialysate, and centrifuge for 10 min in a cold microcentrifuge to remove any precipitate. Transfer the supernatant into a fresh tube, and mix by inverting the tube several times (do not vortex).

14. Snap-freeze in small aliquots at -80°C . Use each aliquot only once, and do not refreeze.
15. The yield should be about 5 mg of protein/roller bottle or L of lymphoblast suspension, at 15–20 mg protein/mL of extract. At least 60 mg of whole-cell extract are needed to make a successful phosphocellulose fraction.

3.2.4. Phosphocellulose Fractionation (CFII)

1. Prepare phosphocellulose P11 (Whatman) according to the manufacturer's instructions. Pour 5 mL into a 1.6-cm diameter column in the cold, and equilibrate with buffer A at a speed of 20 mL/h.
2. Load 60 mg of extract protein, and wash in buffer A until the UV absorbance decreases and is steady (about 2 column-volumes).
3. Elute with buffer A containing 1 M KCl. Collect 1-mL fractions.
4. Pool the peak fractions.
5. Concentrate in an Amicon pressure cell concentrator for about 2–3 h to about 1 mL (*see Note 17*).
6. Dialyze overnight into extract dialysis buffer.
7. Microcentrifuge for 10 min at full speed and aliquot into small (20- μL) amounts and snap-freeze. Store at -80°C . A good CFII should contain 8–15 mg of protein/mL.

3.3. Repair Reactions to Assay for Activity of Purified Proteins

3.3.1. Setting Up Repair Reactions

Carry out all operations on ice.

1. Make a premix A by combining 10 μL /reaction of 5X buffer + 1 μL per reaction of CPK.
2. Make a premix B: 0.2 μL of fresh (<1 wk after activity date) [α - ^{32}P]dATP, 1.5 μL of 1 M KCl, and 7.3 μL of milli-Q water (*see Note 18*). For reactions with CFII_s add ~150 ng of RPA (*see Note 2*). Adjust the KCl and water in premix B to a volume of 9 μL /reaction and the KCl to give 70 mM in a 50- μL reaction, taking into account the concentration of KCl in the extract and other added reagents. Make enough for two more reactions than needed. Carry out all procedures using appropriate radiological protection and monitoring.
3. Place 10 μL of DNA mix (250 ng of each plasmid) in the bottom of a 1.5-mL microcentrifuge tube.
4. Add 11 μL of premix A.
5. Add 9 μL of premix B.
6. Quick-thaw the extracts, and add 200 μg of extract protein, or 100 μg of CFII protein in 15 μL of extract dialysis buffer (*see Note 19*).
7. Add an appropriate amount of purified protein made up to 5 μL in buffer (if different from extract dialysis buffer), or 5 μL of buffer alone (*see Note 20*).
8. Centrifuge for a few seconds to collect the components at the bottom of the tube. Mix by vortexing, centrifuge again, and incubate for 3 h at 30°C in a heat block or water bath.

- 9 Reactions using CFIIIs require the addition of PCNA (*see Note 3*) at this stage. Dilute the stock PCNA in its own buffer, add 25 ng to each reaction, and incubate for a further 10 min at 30°C. A “cold chase” of unlabeled dATP can be added at this stage if background signal in the damaged plasmid is a problem (*see Note 21*).
10. After incubation, stop the reactions by adding EDTA to 20 mM and store at –20°C or below.

3.3.2. Working Up Repair Reactions

1. To each reaction add 2 µL of RNase A (80 µg/mL final), and incubate at 37°C for 10 min.
2. Add 3 µL of SDS (0.5% final concentration) and 6 µL of proteinase K (190 µg/mL final concentration) to each tube, mix, and incubate at 37°C for 30 min.
3. Extract the mixture with 50 µL of phenol:chloroform:isoamyl alcohol (25:24:1), and microcentrifuge at maximum speed for 5–10 min.
4. Remove the aqueous phase, and transfer to a fresh tube containing 25 µL 7.5 M ammonium acetate. Mix and add 160 µL of absolute ethanol. Mix, and chill the tubes on dry ice for 15–30 min.
5. Microcentrifuge for 10 min at 4°C. Remove the supernatant with an ultrafine gel-loading tip, avoiding the DNA pellet. Add 200 µL of 70% ethanol, spin, and remove as much ethanol as possible.
6. Dry the DNA pellet in a Speed Vac for about 5 min until the liquid is removed, but do not overdry.
7. Linearize the DNA with an appropriate restriction enzyme. pBS and pHM14 have a unique *Bam*HI site and 10 U are used/50 µL reaction in the manufacturer’s buffer. Incubate for 60 min at 37°C.
8. Add loading dye to the tubes, mix and load onto a 0.8% agarose gel in 1X TBE, with both the gel and buffer containing 0.25% ethidium bromide. Run the samples into the gel at 100 V for 15 min and then at 40 V overnight.
9. Photograph the gel with UV-B transillumination using Polaroid type 57 film (f11, 1 s), and type 55 film (f8, 30 s) to obtain a negative for quantification.
10. Dry the gel onto Whatman 3MM paper under vacuum at 80°C for about 1.5 h.
11. Put the dried gel into a cassette with intensifying screens and expose to preflashed X-ray film (*see Note 22*) and place at –80°C. Expose the film for 3–6 h for extracts and 6 h to overnight for CFIIIs. (*See Fig. 2.*)

Alternatively, put the gel into a phosphorimager cassette, and read using the manufacturer’s instructions.

3.3.3. Analysis of Results

1. Excise the band from the exposed gel (together with the attached filter paper) under UV light, and obtain the cpm in a scintillation counter. Cpm can also be obtained by using an appropriately calibrated phosphorimager.
2. Measure the relative densities of the bands of both plasmids on the negative of the photograph of the ethidium bromide-stained gel.

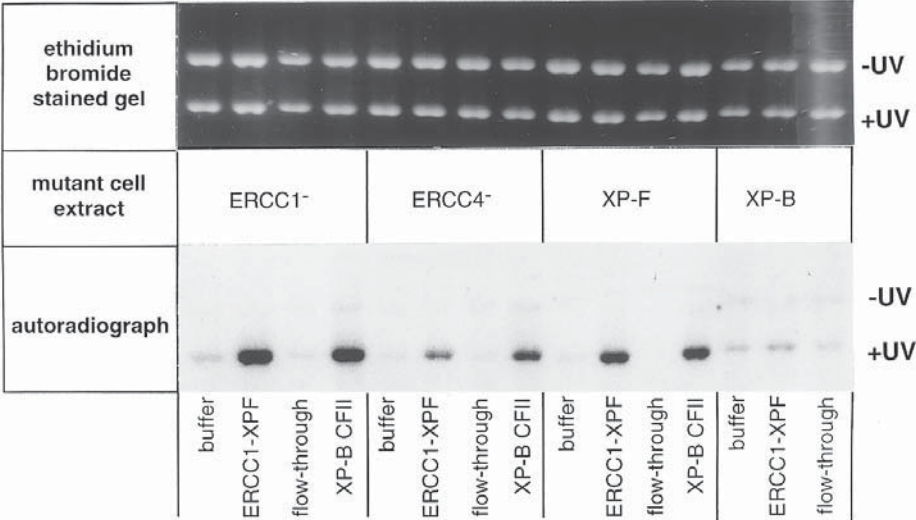


Fig. 2. Repair synthesis assay for the detection of ERCC1-XPF complex in pooled fractions from a mono S column. Pooled fractions from a mono S FPLC column were assayed for complementation of different excision repair mutant CFIIIs from the following cells: Chinese hamster ovary 43-3B (ERCC1⁻); CHO UV41 (ERCC4⁻, equivalent to XPF); human XP-F; and CHO 27-1 (XPB). Each CFII was supplemented with Mono S buffer alone (buffer), pooled Mono-S fractions (ERCC1-XPF), flowthrough from the Mono S column (flowthrough), and a CFII from the mutant CHO 27-1 (XP-B CFII). A total of 100 µg of CFII protein or CFII + complementing protein were added in each lane to 250 ng of each of UV-damaged and undamaged plasmid DNA, supplemented with RPA and PCNA as described in the text.

3. Calculate the fmol of dAMP incorporated/reaction by dividing the cpm by 10 and by the correction factor for the activity date of the isotope, as read from a ³²P decay table. Normalize this for DNA recovery by dividing by the relative amount of DNA obtained from the density measurement of the ethidium bromide-stained gel (see Note 23).

4. Notes

1. The endonuclease III enzyme is expressed from *E. coli* and purified exactly according to the method of Asahara et al. (22). It will be quite concentrated after purification (10–20 mg/mL) and needs to be titrated to establish the correct amount to use. Too much enzyme may cleave unirradiated DNA. It is important to inactivate the enzyme at 65°C for 5 min with gentle shaking when large volumes are being treated to prevent excess plasmid nicking. Titration of the enzyme can be carried out using 250 ng of irradiated plasmid (450 J/m²) mixed with 250 ng of unirradiated plasmid. Add 1 µL of 10X Nth buffer and 1 µL of

diluted enzyme and distilled water to a final volume of 10 μL . Incubate for 30 min at 37°C, and stop the reaction by heating to 65°C. Add loading buffer containing 1% SDS and examine by electrophoresis through a 0.8% agarose gel containing 0.25% ethidium bromide until separation of nicked (form II) and closed-circular plasmid DNA (form I) is evident (approx 4 h at 75 V for a $15 \times 10 \text{ cm}^2$ gel). Photograph the gel with Polaroid type 55 film and scan the negative by densitometry to quantify each band. The nicked DNA has a higher molar fluorescence than closed-circular DNA by a factor of about 1.6 (this can vary for the staining conditions and plasmid used). Calculate the fraction of nicked molecules for each plasmid by the formula:

$$\text{Nicked fraction} = (\text{area form II}/1.6) / [(\text{area form I}) + (\text{area form II}/1.6)] \quad (1)$$

The average nicks per molecule can then be calculated from the nonnicked fraction ($1 - \text{nicked fraction}$) by the Poisson distribution, where the average nicks per circle = $-\ln(\text{nonnicked fraction})$. Make a plot of the amount of enzyme vs average nicks per circle in UV-irradiated and nonirradiated DNA. Identify the amount of enzyme for which UV-specific nicking is complete, but for which minimal nicking of unirradiated DNA occurs. Use this dilution for the large plasmid preparation.

2. Human RPA can be produced as a recombinant protein in *E. coli* and purified according to the method of Henricksen et al. (23). The normal yield is about 500 $\mu\text{g}/\text{mL}$ in extract dialysis buffer containing 250 mM KCl. It is stored in aliquots at -80°C .
3. Recombinant human PCNA can be purified from an *E. coli* expression strain on Q-Sepharose, S-Sepharose, hydroxyapatite, and phenyl-Sepharose as described by Fien and Stillman (24), except for the procedure for the final column. Protein is equilibrated and loaded onto the phenyl Sepharose column in buffer containing 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.01% NP-40, 10% glycerol, 2 mM benzamidine, 2 μM pepstatin A, 10 mM NaHSO_3 , 1 mM PMSF, and 5 mM DTT containing 1.0 M ammonium sulfate. The protein is eluted from the column using a gradient in this buffer from 1.0 M ammonium sulfate and 0% ethylene glycol to 0 M ammonium sulfate and 50% ethylene glycol. PCNA elutes from the column in 0.35 M ammonium sulfate/32.5% ethylene glycol. At the end of this procedure, PCNA should be a single homogenous band of 36 kDa as detected by Coomassie blue staining of an SDS-polyacrylamide gel. The final protein concentration after dialysis into extract dialysis buffer is about 600 $\mu\text{g}/\text{mL}$.
4. Any plasmids are theoretically suitable, but to obtain a high yield of DNA it is most convenient to use plasmids with relaxed copy control, such as those derived from pUC vectors. We use the 2.9-kb pBluescript KS⁺ (pBS, Stratagene) as the irradiated plasmid, and the same plasmid with a 0.8-kb insert in the *Eco*R1 site, pHM14 (3.7 kB) as the unirradiated control. Use of an *E. coli* strain that contains the *recA* mutation may help limit the formation of plasmid multimers. JM109 is a widely available *recA* strain that also contains an *endA* mutation which helps to reduce degradation of the plasmid DNA during cell lysis. pBS and pHM14

plasmids prepared from stocks of transformed JM109 cells stored at -20°C or -80°C can form dimers, which cannot be used in the repair assay. The way to avoid this is to use fresh transformants from a freshly plated-out culture.

5. Typical yields are 2 mg of plasmid/L of culture for the pBS plasmids. At least 10 mg of UV-irradiated plasmid are needed at this stage, i.e., 5 L of culture. This is because subsequent treatment by Nth protein nicks UV-irradiated DNA at pyrimidine hydrates and the nicked DNA is separated from closed-circular by cesium chloride density gradient centrifugation. Since only closed-circular DNA is used in the repair assay, up to 60% of the UV-irradiated plasmid is removed. Six milligrams of the unirradiated plasmid are sufficient.
6. UV-C irradiation at 254 nm produces roughly 1 pyrimidine dimer photoproduct (about 0.75 cyclobutane pyrimidine dimer and 0.25 [6–4] photoproduct)/1000 bp per 100 J/m^2 . A relatively low fluence rate ensures a more even irradiation of the whole DNA sample.
7. Cesium chloride gradients are set up essentially as described in the *Current Protocols in Molecular Biology* manual (25). We centrifuge in a Ti60 rotor at 38,000 rpm to achieve 145,000g. All operations are carried out in the dark when the DNA is in contact with ethidium bromide to reduce damage to the DNA. Wear gloves, face shield, and protective clothing when handling ethidium bromide, and dispose of it according to local safety regulations.
8. Pumping the solutions to the bottom of the tubes is best achieved using glass capillaries attached to the end of the pump tubing. These are placed in the gradient tubes and can be removed at the end by lifting them out smoothly and gently without disturbing the gradient.
9. When the DNA is loaded onto the gradient, it should be visible as a distinct layer. If it starts to sink into the gradient, this indicates that there is still some cesium chloride present in the DNA. The time of centrifugation is dependent on the plasmid and the density of the solutions. For our plasmids, 19 h give good separation of nicked and supercoiled DNA.
10. When loading aliquots of fractions onto an agarose gel, there is no need to add loading solution containing dye to every fraction, since the sucrose in the fractions makes them dense enough to stay at the bottom of the wells.
11. We have prepared extracts from as few as 10^8 cells, but starting with 10^9 cells is much easier and more likely to be successful. Cells that are overgrown yield extracts with very poor repair activity. The viability of the cells should be measured by exclusion of a dye, such as trypan blue or nigrosin, and should be 98% or greater. Fibroblast cells should be just confluent when harvested, since they should be as healthy as possible for good extracts.
12. These methods for whole cell extract preparation are adapted from those of Manley et al. (26,27).
13. The cells should stay intact during incubation with hypotonic lysis buffer and only break during Dounce homogenization.
14. After about half of this is added, the viscosity increases dramatically. Stir very slowly to avoid shearing the DNA (about 1 turn/s). Continue stirring for about 30 min.

15. The resulting solution is very viscous and needs to be poured into the tubes gently. This may take a little practice!
16. The last 1 mL is usually viscous, because it contains DNA that has not fully pelleted. If a fixed-angle rotor, such as a Ti60, is used, more care must be taken to avoid mixing the DNA with the supernatant.
17. Concentrate the fractions in 1 M KCl before dialysis into 100 mM KCl, since this helps to prevent precipitation and loss of protein.
18. The amount of KCl or NaCl in a reaction should be 70 mM. The repair levels are considerably reduced at levels higher than this, and specificity for NER is reduced at lower salt. The extract dialysis buffer contains 100 mM KCl. When adding purified proteins to extracts, take into account the amount of salt in the protein buffer, and adjust the amount of KCl in premix B to make the final concentration in the 50 μ L repair reaction 70 mM.
19. It is important to add enough total extract or CFII protein to a reaction. Do not add <50 μ g of CFII or 100 μ g of whole-cell extract protein to a 50- μ L reaction, since the signal will be too weak.
20. Titrate the protein added to the mutant extract or CFII to find the optimal activity. Always add buffer alone to the reactions as a control.
21. A chase of unlabeled dATP can be added about 2 min after the PCNA pulse to reduce the background further if this is a problem. However, this can reduce the specific signal if added too soon, so time-points must be taken to establish exactly the amount and when to pulse. Stop the reactions by immersing the tube in dry ice.
22. The film should be preflashed so that the response of the film will be as linear as possible for quantification.
23. Addition of pure proteins to CFIs in repair reactions requires careful quantification in order to establish the contribution of the protein to the reaction as a whole. The first step is to determine the counts per minute in each band of interest. Scintillation counting of the gel can be carried to high efficiency without solubilizing it when ^{32}P has been used as a label. The bands remain stained with ethidium bromide in the dried gel, and guidelines for cutting the bands can be drawn while illuminating with UV-A or UV-B light (wear suitable eye protection and cover exposed skin). Under standard conditions described for the repair reactions, each 50- μ L reaction mixture contains 2 μCi of [α - ^{32}P] dATP at 3000 Ci/mmol and 8 μM cold dATP, so that there are 11 dpm/fmol of dATP. With a counting efficiency of 90%, this gives about 10 counts/min/fmol of dATP. Under these particular conditions, fmol of dAMP incorporated/reaction can be calculated by dividing the counts/min by 10 and by the correction factor for the activity date of the isotope as read from a ^{32}P decay table. This value should in turn be normalized for any differences in DNA recovery between the tracks.

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Dual-Incision Assays for Nucleotide Excision Repair Using DNA with a Lesion at a Specific Site

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1. Introduction

Cells remove a wide array of potentially toxic and mutagenic lesions from their genomes by a major repair pathway called nucleotide excision repair (NER). This repair process involves a multiprotein nuclease complex that incises a damaged DNA strand on the 5'- and 3'-sides of a lesion (**1**). In humans, defects in the genes that participate in NER can lead to a rare recessive disorder, xeroderma pigmentosum (XP). Hypersensitivity to sunlight and a predisposition to skin cancer are the most characteristic traits of XP patients. NER-defective XP cells belong to one of seven genetic complementation groups (A–G). Although the NER process in mammalian cells consistently leads to the excision of damaged DNA fragments 24–32 nucleotides in length, the exact positions of the 5'- and 3'-incisions depend on the lesion being repaired. For instance, during the removal of a thymine dimer, incisions are made at the 22nd–24th phosphodiester bond on the 5'-side of the lesion and at the 5th phosphodiester bond on the 3'-side of the lesion (**2**). The main sites of incision during repair of DNA containing a 1,3-intrastrand d(GpTpG)-cisplatin crosslink are at the 16th–20th phosphodiester bond 5'- and the 8th–9th phosphodiester bond on the 3'-side of the lesion (**3**).

The core factors that participate in NER in eukaryotes have been identified using a combination of biochemical and genetic approaches (**4,5**). Analysis of the mechanism of NER using cell-free extract systems and purified proteins requires suitable DNA substrates containing characterized DNA lesions. For

certain mutagens, sufficiently defined DNA substrates can be obtained after globally damaging DNA with UV light or using chemicals that specifically make certain bulky adducts, such as thymine-psoralen mono-adducts, cisplatin-DNA intrastrand crosslinks, or acetylaminofluorene-DNA adducts. In this case, repair DNA synthesis can be monitored as an end point by measuring incorporation of radiolabeled deoxynucleotides during repair of the damaged DNA (*see* Chapter 29).

In many instances, more detailed information is required. This can be obtained by using DNA substrates containing chemically defined lesions that are placed at a unique site in a DNA duplex. In this way, NER can be readily and specifically measured by detecting the 24–32 nucleotide products of the dual-incision reaction. This is a simpler reaction than the full repair process, since it does not involve a eukaryotic DNA polymerase holoenzyme. Moreover, the production of 24- to 32-mers is very specific for NER, so that there can be no confusion with signals arising from other repair pathways, such as base excision repair or mismatch repair. One direct method is to construct DNA substrates containing a single defined lesion in a linear duplex of sufficient size, usually in the middle of a 140–150-mer. This is obtained by ligating together a short oligonucleotide containing the lesion with a series of complementary and overlapping oligonucleotides. An internal radiolabel is placed near the lesion, so that excised fragments can be detected. This approach has been well described elsewhere (6,7). An alternative procedure is to place the lesion at a specific site in a covalently closed-circular DNA molecule containing a lesion. Closed-circular plasmid molecules are useful for a number of purposes in biological systems, including studies of DNA repair and mutagenesis (8). Circular duplexes also can be used to study the effects of nucleosome structure on repair (9,10), and the coupling of chromatin assembly to NER, where chromatin assembly propagates from a site of repair (11,12). Circular duplexes can be constructed with or without an internal radiolabel. The advantage of an internal label is that repair can be detected directly after electrophoresis, and quantification of repair is straightforward. The disadvantage is that the labeled substrate must be used within 1 or 2 wk of construction because of radioactive decay.

This chapter describes several methods for detection of repair of a specific lesion in closed-circular DNA. As a model lesion, we use the 1,3-intrastrand d(GpTpG)-cisplatin crosslink. This adduct has two main advantages. First, it is exceptionally well repaired by the mammalian NER system (3,13–15). Second, with care it is possible for any laboratory to construct DNA modified in this way, without specialized tools or reagents.

Three methods are given for analysis of repair. One is to incorporate a radioactive label internally near the lesion and measure excision by detecting radioactive excised oligomers. Two other methods use DNA that is not internally

labeled. The advantage of this approach is that a large amount of specifically modified DNA substrate can be prepared and stored frozen, and then used when convenient. The first method for detection of repair of such unlabeled DNA is to detect repair products with a labeled complementary oligonucleotide by a Southern blot method. The second, more recently developed method is to 3'-end-label the excised oligonucleotide directly with radiolabeled dNTP and a DNA polymerase, using a complementary oligonucleotide with a 5'-overhang that serves as a template. This protocol has the advantage of being the fastest and probably the most sensitive method of detection, but it relies on accurate foreknowledge of the site of 3'-incision for the particular lesion being used.

2. Materials

2.1. Cisplatin-Adducted Oligonucleotide

1. *cis*-Platinum(II)diamminedichloride (cisplatin; Sigma).
2. 2X Platination buffer: 6 mM NaCl, 1.0 mM Na₂HPO₄, 1.0 mM NaH₂PO₄.
3. T4 Polynucleotide kinase (10,000 U/mL) and 10X kinase buffer (New England Biolabs; NEB).
4. 5 M NaCl.
5. Thin-layer chromatography (TLC) plates suitable for UV shadowing analysis (silica gel; Merck F254 or Polygram CEL 300 PEI/UV₂₅₄—20 × 20 cm²).
6. Sephadex G25.
7. [γ -³²P] ATP (>5000 Ci/mmol; Amersham).
8. Sequencing gel solutions: Concentrate, diluent, and buffer (Sequagel, National Diagnostics) are mixed according to the manufacturer's instructions to make 12–20% gels. To 100 mL of the above mixture add 0.32 mL of 25% ammonium persulfate and 40 μ L of TEMED to the gel components prior to pouring the gel. Use appropriate spacers to form either 0.4- or 1.5-mm thick gels.
9. 10X TBE: 108 g of Tris base, 55 g of boric acid, 40 mL of 0.5 M EDTA, pH 8.0, and deionized water to a final volume of 1 L.
10. Sequencing gel-loading buffer : 0.9 mL deionized formamide mixed with 0.1 mL of dye solution (10X TBE, 0.25% bromophenol blue and 0.25% xylene cyanol); 1.0-mL aliquots can be stored frozen (–20°C).
11. Intensifying screens for Kodak X-ray film (XOMAT AR or BioMax MS) or phosphorimager plates (Molecular Dynamics).

2.2. Closed-Circular DNA Containing Cisplatin-Adduct: Radiolabeled and Nonradiolabeled

1. Single-stranded form (+ strand) of bacteriophage M13mp18 DNA (modified as in **Subheading 3.2.**).
2. T4 DNA polymerase (5,000 U/mL, HT Biotechnology; or 3000 U/mL, NEB).
3. T4 DNA ligase (400,000 cohesive end U/mL, NEB).
4. Recombinant T5 exonuclease (**16**) (Amersham).
5. Sephacryl S-400 (Pharmacia).

6. 10X annealing/complementary strand synthesis/ligation buffer: 100 mM Tris-HCl, pH 7.9, 500 mM NaCl, 100 mM MgCl₂, and 10 mM dithiothreitol (DTT) (see **Note 1**).
7. Deoxyribonucleotides (dNTPs) and ribonucleotides (NTPs) (Pharmacia).
8. Nuclease-free bovine serum albumin (BSA) (10 mg/mL) (Gibco BRL or NEB).
9. Restriction endonuclease *Apa*I and 10X restriction endonuclease buffers (NEB buffer 4).
10. Agarose (Gibco BRL).
11. Ethidium bromide (EtBr; 10 mg/mL, Bio-Rad).
12. Cesium chloride (CsCl, Gibco BRL).
13. Quick-Seal polyallomer tubes (2 mL, 11 × 32 mm, Beckman # 344625).
14. H₂O-saturated butanol.
15. Centricon 100 ultrafiltration units.
16. TE: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

2.3. Southern Blot Method

1. 5X Repair reaction buffer: 200 mM HEPES-KOH, pH 7.8, 25 mM MgCl₂, 2.5 mM DTT (Sigma), 10 mM ATP, 110 mM phosphocreatine (di-Tris salt, Sigma), 1.8 mg/mL BSA (nuclease-free, Gibco BRL). The pH of the final repair reaction mixture should be 7.8. Aliquots should be stored at -80°C. (See **Note 2**.)
2. Creatine phosphokinase (CPK, rabbit muscle; Sigma) 2.5 mg/mL in 10 mM glycine, pH 9.0, 50% glycerol. Store aliquots at -20°C.
3. 1 M KCl.
4. 0.5 M EDTA, pH 8.0.
5. RNase A (2 mg/mL).
6. Sodium dodecyl sulfate (SDS), 10 and 20% (w/v).
7. Proteinase K (2 mg/mL).
8. Phenol:chloroform:isoamyl alcohol (25:24:1).
9. 7.5 M ammonium acetate.
10. Glycogen (20 mg/mL; Boehringer Mannheim).
11. Yeast tRNA (1–2 mg/mL).
12. Absolute ethanol (-20°C).
13. 70% Ethanol (-20°C).
14. Restriction endonucleases: *Hind*III, *Xho*I, and 10X restriction endonuclease buffer (NEB buffer 2).
15. TLC plates: see **item 5, Subheading 2.1**.
16. Sequencing gel solutions, 10X TBE and sequencing gel loading buffer (see **items 8–10, Subheading 2.1**).
17. Hybond N+ DNA transfer membrane (Amersham).
18. Whatman 3MM paper.
19. 20X SSC: 3 M sodium chloride, 0.3 M trisodium citrate, pH to 7.0 with 1 N HCl.
20. Hybridization buffer (per 200 mL): 70 mL of 20% SDS, 80 mL of 25% PEG 8000, 10 mL of 5 M NaCl, 26 mL of 1 M potassium phosphate buffer, pH 7.0, 14 mL of H₂O.

2.4. End-Labeling Dual-Incision Products

1. 5X Repair reaction buffer: *see* item 1, Subheading 2.3.
2. CPK (*see* item 2, Subheading 2.3.).
3. Restriction endonucleases (*Hind*III, *Xho*I) and 10X restriction endonuclease buffer (NEB buffer 2).
4. Sequencing gel solutions, 10X TBE and sequencing gel-loading buffer (*see* items 8–10, Subheading 2.1.).
5. Sequenase enzyme (Sequenase™ v2.0; 13 U/μL; Amersham) and Sequenase dilution buffer.
6. *Escherichia coli* DNA polymerase I (Klenow fragment) and 10X DNA polymerase buffer (Boehringer Mannheim).
7. *Msp*I-digested pBR322 DNA (NEB).
8. [α -³²P] dCTP (3000 Ci/mmol; Amersham).

3. Methods

3.1. Synthesis, Purification and Characterization of an Oligonucleotide Containing a Single 1,3-Intrastrand d(GpTpG)-Cisplatin Crosslink

1. Purified 24-mer oligonucleotide containing a unique GTG sequence (5'-TCTTCTTCTGTGCACTCTTCTTCT-3') is allowed to react at a concentration of 1 mM with a threefold molar excess of cisplatin (3 mM) for 16 h at 37°C in a buffer containing 3 mM NaCl, 0.5 mM Na₂HPO₄, and 0.5 mM NaH₂PO₄ (17), as follows: Dissolve cisplatin in 2X platination buffer to a final concentration of 6 mM. Dissolve the 24-mer oligonucleotide at a concentration of 2 mM in water, and add an equal volume of this solution to the buffered cisplatin solution. Also perform a mock platination reaction using 2X platination buffer without cisplatin. Incubate in the dark at 37°C for 16 h. Use reaction volumes <100 μL to facilitate purification in step 2. (*See* Note 3.)
2. Stop the platination reaction by adding NaCl to 500 mM. Purify the platination reaction mixture on a 1-mL Sephadex G25 spin column equilibrated in H₂O.
3. Dilute a 1-μL aliquot of the purified platination reaction mixture (and also the mock-treated control oligonucleotide reaction mixture) in water to 20 pmol/μL (assume 100% recovery). Perform 5'-³²P-phosphorylation analysis by setting up a reaction mixture containing 1 μL (20 pmol) of platinated (or mock-treated) oligonucleotide, 1 μL of 10X T4 polynucleotide kinase buffer, 4 μL of 100 μM ATP, 0.2 μL of [γ -³²P] ATP (>5000 Ci/mmol), 1 μL of T4 polynucleotide kinase (10 U/μL), and 2.8 μL of H₂O. Incubate at 37°C for 1 h, and stop the reaction by incubating at 68°C for 15 min.
4. Add 8 μL of sequencing gel-loading buffer to each 10 μL of reaction. Heat the samples at 95°C for 3 min, and place immediately on ice before loading on a 0.4-mm thick denaturing 20% polyacrylamide gel (40 cm long). Run the gel in 1X TBE buffer until the xylene cyanol dye-front is approx 28 cm from the wells (the dyes have mobilities on a 20% polyacrylamide gel such that xylene cyanol

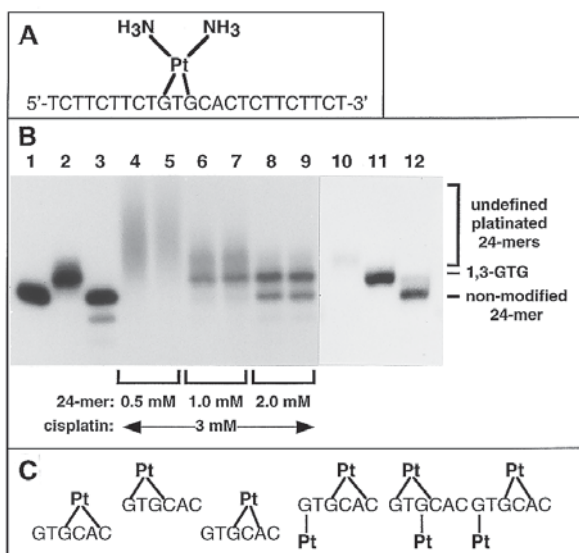


Fig. 1. (A) 24-mer oligonucleotide containing a single 1,3-intrastrand d(GpTpG)-cisplatin crosslink. (B) ³²P-labeled platinated and nonmodified oligonucleotides were separated in a denaturing 20% polyacrylamide gel. The 1,3-intrastrand crosslinked platinated oligonucleotide (lane 2) is observed as a single band whose mobility is retarded by approximately one nucleotide relative to the nonmodified oligonucleotide (lane 1). Lane 3 contains nonmodified 24-mer (HPLC-purified) used for the platination reactions in lanes 4–9. Platination reactions using a 3:1 molar ratio of cisplatin to oligonucleotide lead to the platination of almost all the available oligonucleotide (lanes 6 and 7), although the purest preparation of oligonucleotide containing a single 1,3-intrastrand d(GpTpG)-cisplatin crosslink was obtained using a 3:2 molar ratio of cisplatin to oligonucleotide (lanes 8 and 9), because overplatinated DNA products form a smear above the desired product (lanes 4 and 5). DNA was eluted from gel slices containing either the smear of undefined platinated 24-mers (lane 10), the defined 1,3-GTG platinated 24-mer (lane 11), or nonmodified 24-mer (lane 12). (C) The probable identity of platinated DNA products present in the smear includes an assortment of monoadducts, 1,3- and 1,4-intrastrand crosslinks.

- migrates as a 29-mer oligonucleotide and bromophenol dye migrates as a 9-mer oligonucleotide). Expose the gel (without fixing or drying) to X-ray film for 1 h. The 1,3-intrastrand crosslinked platinated oligonucleotide is identified as a single band whose mobility is retarded by approximately one nucleotide relative to the nonmodified oligonucleotide (**Fig. 1**; see **Notes 4 and 5**).
- After analysis of a small aliquot of each platination reaction (as described in **steps 3 and 4**), a preparative denaturing polyacrylamide gel (20%; 1.5 mm thick; 40 cm long) is used to purify the remainder of each reaction. Mix each purified

- platination reaction mixture with 0.8 vol of sequencing gel-loading buffer (without dyes; *see* **Note 6**). Heat the samples at 95°C for 3 min, and place immediately on ice before loading the gel. Load sequencing gel-loading buffer with dyes in adjacent lanes to follow the migration during electrophoresis. Run the gel in 1X TBE buffer until the xylene cyanol dye-front is approx 28 cm from the wells.
6. Place the appropriate region of the preparative gel onto a TLC plate. Visualize the oligonucleotides using a handheld UV lamp (254 nm; *see* **Note 7**), excise the desired platinated and nonplatinated oligonucleotides (use a clean scalpel for each sample) and place in a microcentrifuge tube (*see* **Note 8**). Crush or finely slice each polyacrylamide fragment before resuspending in 0.5–1.0 mL of H₂O. Incubate at 37°C for 16 h with agitation. A rapid freeze–thaw step may improve recovery. Centrifuge the samples in a microcentrifuge to pellet the polyacrylamide fragments and recover the eluted oligonucleotide. It is convenient to lyophilize this solution to approx 100 μ L for purification on a 1-mL Sephadex G25 spin column. Alternatively, the oligonucleotide can be recovered by ethanol precipitation or with a Microcon-30 concentrator. Quantify an aliquot of each sample by spectrophotometry. (*See* **Note 9**).
 7. Repeat the 5'-³²P-phosphorylation analysis described in **steps 3** and **4** for 20 pmol of each gel-purified oligonucleotide to determine the purity (*see* **Note 10**). Purified platinated oligonucleotides can be stored lyophilized or in TE buffer for several years at –80°C. Before use in the construction of closed-circular duplex DNA substrates (described in **Subheading 3.2.**), it is necessary to 5'-phosphorylate the platinated oligonucleotides from **step 6** with T4 polynucleotide kinase and ATP.

3.2. Construction of closed-Circular Duplex DNA Containing a Single 1,3-Intrastrand d(GpTpG)-Cisplatin Crosslink

A fivefold molar excess of 24-mer platinated oligonucleotide containing a single 1,3-intrastrand d(GpTpG)-cisplatin crosslink (described in **Subheading 3.1.**) is annealed to the single-stranded form (+ strand) of bacteriophage M13mp18 DNA modified to contain a sequence complementary to the platinated oligonucleotide within the polycloning site (**3,18**). The 3'-terminus of the oligonucleotide acts as a primer for complementary strand synthesis by T4 DNA polymerase. The newly synthesized DNA strand is covalently closed with T4 DNA ligase resulting in a circular DNA duplex containing the single cisplatin-DNA adduct at a specific site (**Fig. 2A**).

1. For the annealing reaction, make a mixture containing 50 μ L (25 μ g) of single-stranded M13 DNA (500 ng/ μ L), 3.8 μ L (380 ng) of 5'-phosphorylated 24-mer oligonucleotide containing a 1,3-intrastrand cisplatin crosslink (100 ng/ μ L), 7.5 μ L of 10X annealing/complementary strand synthesis/ligation buffer and 13.7 μ L of H₂O. Incubate the mixture at 65°C for 5 min, 37°C for 30 min, 25°C for 20 min, and finally 4°C for 20 min.
2. For the complementary strand synthesis/ligation reaction prepare a mixture on ice containing 75 μ L of annealed DNA, 12.5 μ L of 10X annealing/complemen-

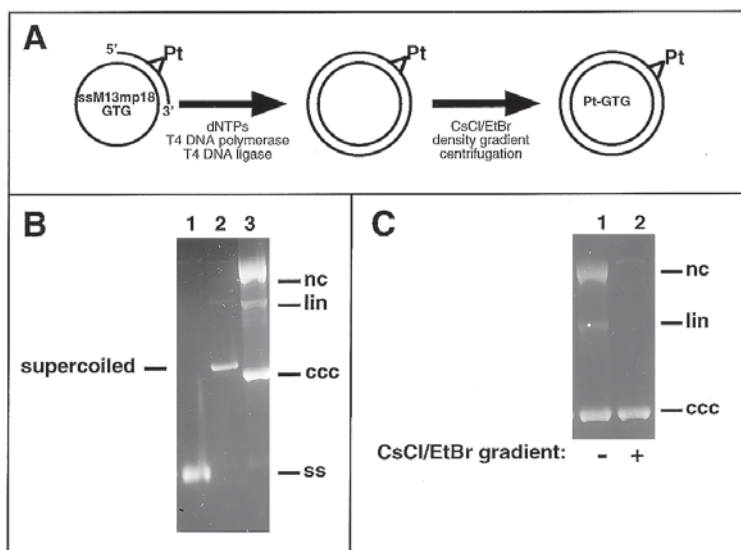


Fig. 2. (A) 24-mer platinated oligonucleotide containing a single 1,3-intrastrand d(GpTpG)-cisplatin crosslink (described in **Subheading 3.1.**) is annealed to the single-stranded form (+ strand) of bacteriophage M13mp18 DNA modified to contain a sequence complementary to the platinated oligonucleotide within the polycloning site (ssM13mp18GTG). The 3'-terminus of the oligonucleotide acts as a primer for complementary strand synthesis by T4 DNA polymerase. The newly synthesized DNA strand is covalently closed with T4 DNA ligase resulting in a circular DNA duplex containing the single cisplatin–DNA adduct at a specific site. Closed-circular DNA (Pt-GTG) is then purified using CsCl/EtBr gradient centrifugation. A control DNA substrate is prepared in the same way, except that a nonmodified 24-mer oligonucleotide is used as a primer. (B) Typical reactions (lane 3) result in the conversion of almost all single-stranded DNA (ss, lane 1) into either covalently closed-circular (ccc, ~65% of molecules), linear (lin, ~15%), or nicked-circular DNA (nc, ~20%). Lane 2 contains the supercoiled replicative form of M13mp18GTG. The % yield of each form of DNA was quantified by densitometry correcting for the 1.6-fold increased fluorescence of linear and nicked-circular DNA. (C) More than 95% of the DNA substrate is in the closed-circular form after CsCl/EtBr density gradient centrifugation (lane 2).

tary strand synthesis/ligation buffer, 2 μ L of 10 mg/mL BSA, 4 μ L of 100 mM ATP, 12 μ L of 10 mM dNTP mixture (contains 10 mM each of dATP, dCTP, dGTP, TTP), 15 μ L of T4 DNA polymerase, 4 μ L of T4 DNA ligase, and 75.5 μ L of H₂O. Incubate at 37°C for 3 h (see **Note 11**).

- Analyze the reaction products (remove 1- μ L aliquots from 200 μ L reactions) using a 0.8% agarose gel run in 1X TBE buffer at 50 V for 16 h (**Fig. 2B**). Add EtBr (0.25 μ g/mL) to the gel and running buffer. It is useful to load single-

stranded DNA to assess the efficiency of complementary strand synthesis. Typical reactions result in the conversion of almost all single-stranded DNA into either closed-circular (~65% of molecules), linear (~15%), or nicked-circular DNA (~20%) (see **Fig. 2B**, lane 3; see **Note 12**).

4. Removal of nicked and linear DNA forms from closed-circular DNA can be achieved using CsCl/EtBr density gradients with a final CsCl concentration of 1.55 g/mL. For 8 × 2.0 mL gradients dissolve 15 g of CsCl in 9.5 mL of H₂O at 37°C for 1 h. Aliquot 1.352 mL of this solution into 2.2-mL microcentrifuge tubes. Dilute the DNA samples (+/-*Apa*LI digestion; see **Note 11**) to a final volume of 500 µL with TE buffer, and add to the 2.2 mL tubes containing CsCl solution. Transfer the DNA/CsCl solution to 2-mL Quick-Seal polyallomer tubes using a wide-bore needle and syringe. Cover each tube with foil, and add 148 µL of 10 mg/mL EtBr solution to each Quick-Seal tube using a fine pipet tip. Heat-seal the polyallomer tubes under dim light. Perform CsCl/EtBr density gradient centrifugation at 85,000 rpm in a TLA100.2 (Beckman) ultracentrifuge rotor (315,000g) at 18°C for 24 h (see **Note 13**).
5. Unload the centrifuge tubes very carefully from the rotor in a dark room. Use a handheld UV lamp (312 nm) to visualize closed-circular DNA. Use a wide-bore needle and 1-mL syringe to remove this DNA in approx 500 µL of solution. It is important to keep the DNA away from light until all traces of EtBr have been removed to avoid nicking. Add an equal volume of H₂O-saturated butanol, vortex for 5 s, and centrifuge for 1 min. Remove and discard the upper phase (pink owing to EtBr). Repeat the extractions until no traces of EtBr remain in either phase. Remove as much butanol as possible after the final extraction, and dilute the DNA solution to 2.0 mL using TE buffer.
6. Place each sample in a Centricon 100 or Centricon 30 ultrafiltration unit, and centrifuge at 1000g for 30–60 min at 4°C to concentrate the DNA to approx 50 µL. Add more TE buffer to 2.0 mL, and repeat this step four more times (see **Note 14**). Quantify the final DNA solution using a spectrophotometer and confirm the DNA purity using the gel electrophoresis conditions described in **step 3**. The purified DNA may be stored in aliquots at –80°C for more than 1 yr (see **Note 15**).

3.3. Construction of Internally Radiolabeled Closed-Circular Duplex DNA Containing a Single 1,3-Intrastrand d(GpTpG)-Cisplatin Crosslink

1. Radiolabel the gel-purified 24-mer oligonucleotide containing the 1,3-intrastrand d(GpTpG)-cisplatin crosslink (**Subheading 3.1.**) at its 5'-end by adding 30 pmol of oligonucleotide (1 µL; 0.25 µg/µL) to a microcentrifuge tube containing 1 µL of 10X T4 kinase buffer, 7 µL of [γ -³²P] ATP (>5000 Ci/mmol) and 1 µL of T4 polynucleotide kinase. Mix the contents and centrifuge. Incubate the reaction mixture at 37°C for 30 min. Supplement the reaction mixture with a cold-chase of 100 µM ATP (1 µL of 1 mM ATP) and 0.2 µL of T4 polynucleotide kinase. After mixing the contents, incubate the reaction mixture at 37°C for a further 30 min.

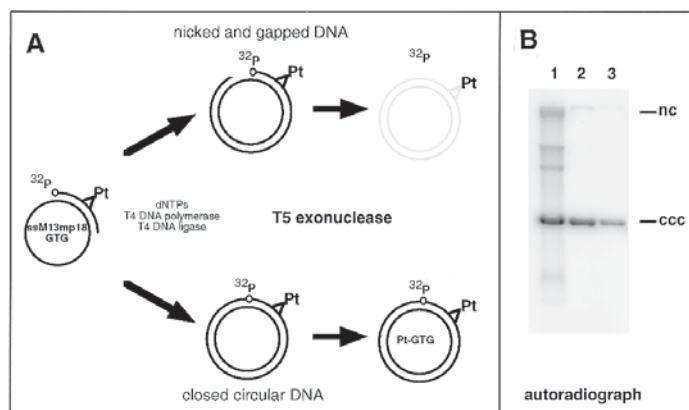


Fig. 3. Production of internally labeled closed-circular DNA containing a 1,3-intrastrand d(GpTpG)-cisplatin adduct. **(A)** Schematic. **(B)** Autoradiograph showing: lane 1, internally labeled products of a reaction with T4 DNA polymerase and T4 DNA ligase; lane 2, reaction mixture after treatment with T5 exonuclease to digest nicked and single-stranded DNA; lane 3, reaction mixture after purification on an S-400 spin column.

- Inactivate the kinase reaction by heating at 95°C for 5 min. The ^{32}P -labeled oligonucleotide can be used as a marker on sequencing gels (*see Note 16*).
- For the annealing reaction, add 10 μL (10 μg ; 4 pmol) of single-stranded M13 DNA and 2.2 μL of 10X annealing/complementary strand synthesis/ligation buffer to the ^{32}P -labeled 24-mer oligonucleotide (11 μL ; 30 pmol). The ratio of oligonucleotide to M13 DNA should be titrated to find conditions that give the highest yield of closed-circular product. Incubate the reaction as described in **step 1, Subheading 3.2**.
 - For the complementary strand synthesis and ligation reaction, add the contents of the annealing mixture (23 μL) to a microcentrifuge tube containing 10 μL of 10X NEB buffer 2, 1 μL of BSA (10 mg/mL), 2 μL of 100 mM ATP, 30 μL of 2 mM dNTP mixture (contains 2 mM each of dATP, dGTP, TTP, and dCTP), 10 μL T4 DNA polymerase (3 U/ μL), 2 μL of T4 DNA ligase, and 22 μL of H_2O . Mix the contents of the tube, and incubate at 37°C for 4 h. Stop the reaction by heating at 75°C for 10 min.
 - The reaction now contains a mixture of complete and incomplete products of complementary strand synthesis (**Fig. 3A**). The latter products are degraded by T5 exonuclease (**16**). Add 1 μL (2 μg) of T5 exonuclease, and incubate at 37°C for 2 h. Stop the T5 exonuclease activity by incubating the reaction mixture at 70°C for 5 min.
 - To remove the T5 exonuclease-degraded products, load the 100- μL reaction mixture onto a spin column containing Sephacryl S-400 equilibrated in TE (*see Note 17*). Centrifuge at 2000 rpm (320g) for 1 min, and collect the eluate (**Fig. 3B**).

6. Confirm the presence of the lesion by digesting the DNA with *Apa*L1 restriction endonuclease (see **Note 11**). Measure the radioactivity in the DNA by Cerenkov scintillation counting. Dilute the DNA to a concentration of 50 ng/ μ L, and store aliquots at -80°C (see **Note 18**).

3.4. Gel Purification of Oligonucleotides and Preparation of Radiolabeled Probes for Southern Blots

The 27-mer oligonucleotide (5'-GAAGAGTGCACAGAAGAAGAGGCCTGG-3') that is used as a probe for Southern blotting should be gel-purified.

1. Pour a denaturing 20% polyacrylamide gel (1.5 mm thick; 40 cm long) with wells of 2–3 cm width.
2. Prerun the gel at 50°C in 1X TBE.
3. Prepare the oligonucleotides (50 μg ; 2.5 μL) diluted 1:1 in loading buffer (2.5 μL) without dyes (bromophenol blue or xylene cyanol; see **Note 6**).
4. Heat-denature the oligonucleotides at 95°C for 3 min and cool on ice. Load 50 μg aliquots of oligonucleotide/lane. To see the oligonucleotide by UV shadowing, ~ 0.5 – 2.0 OD units (~ 10 μg) need to be loaded on the gel. Load a blank lane with sequencing gel-loading buffer containing bromophenol blue and xylene cyanol. Run the gel in 1X TBE at 50°C until the xylene cyanol has migrated two-thirds of the distance from the wells (see **Subheading 3.1., step 4**).
5. Transfer the gel to a TLC plate, and cover with Saran Wrap. The DNA will appear as a shadow under a short-wave UV lamp (see **Note 7**). Mark the desired band, and excise it from the bulk of the gel.
6. Crush or finely slice each polyacrylamide fragment before resuspending in 0.5–1.0 mL of H_2O . Incubate at 37°C for 16 h with agitation. A rapid freeze–thaw step may improve recovery. Centrifuge the samples in a microcentrifuge to pellet the polyacrylamide fragments and recover the eluted oligonucleotide. It is convenient to lyophilize this solution to approx 100 μL for purification on a 1-mL Sephadex G25 spin column. Quantify the DNA concentration by spectrophotometry, and store aliquots at -80°C .
7. To prepare radiolabeled probes, add 1.5 μL of 27-mer oligonucleotide (10 pmol) to 1 μL of 10X kinase buffer, 6.5 μL of γ - ^{32}P -ATP (>5000 Ci/mmol, 10 mCi/mL), and 1.0 μL of T4 polynucleotide kinase (10 U/ μL). The radiolabel should be as fresh as possible. Incubate at 37°C for 60 min, and then inactivate the reaction by incubating at 65°C for 15 min. Increase the volume to 100 μL with TE, and purify on a Sephadex G25 spin column. Store shielded at -20°C until required.

3.5. Analysis of NER DNA Products by the Southern Blot Method

1. Reaction mixtures are set up in 50- μL aliquots. Add 10 μL of 5X repair reaction buffer, 1.0 μL of CPK, 1.5 μL of 1 M KCl, and 7.5 μL of H_2O to a 20- μL mixture comprising cell extract (100–300 μg of protein) and cell extract dialysis buffer (which contains 100 mM KCl). The total salt concentration (either KCl or NaCl) in the reactions is kept to a 70-mM final concentration.

2. Preincubate the reaction mixtures at 30°C for 5 min. Add 10 μL (0.25 μg) of DNA substrate to the reactions and incubate for a further 25–30 min.
3. Add 2 μL of 0.5 M EDTA, pH 8.0, 3 μL of 10% SDS, and 6 μL of proteinase K (2 mg/mL) to the reaction tubes. Mix the contents thoroughly, and incubate at 37°C for 30 min or 56°C for 20 min.
4. Increase the volume to 100 μL with TE, and mix with an equal volume of phenol:chloroform:isoamyl alcohol. Mix thoroughly and centrifuge for 5–10 min at maximum speed.
5. Transfer 75 μL of the aqueous phase to a fresh tube containing 30 μL of 7.5 M ammonium acetate, and either 0.5 μL of glycogen (20 mg/mL) or yeast tRNA (100 $\mu\text{g}/\text{mL}$). Mix well. Add 212 μL of ice-cold absolute ethanol. Mix thoroughly and place on dry ice for 20 min.
6. Centrifuge the samples at maximum speed in a chilled microcentrifuge for 30 min. Using a glass Pasteur pipet with a drawn-out tip, gently remove the alcohol (*see Note 19*).
7. Add 200 μL of chilled 70% ethanol, and centrifuge in a chilled microcentrifuge at maximum speed for 5–10 min. Carefully remove the ethanol and discard.
8. Dry the DNA pellet in a centrifuge under vacuum for 5 min. Add 8 μL of deionized water and dissolve the DNA by incubating the tubes at 37°C for 30–60 min.
9. Make a premixture of *Hind*III/*Xho*I enzyme such that each tube will contain 1.0 μL 10X restriction endonuclease buffer, 0.4 μL of H_2O , and 0.3 μL each of *Hind*III and *Xho*I. Add 2 μL of this premix to the DNA, mix, and incubate at 37°C for 60 min (*see Note 20*).
10. Add 8 μL of loading buffer. The samples can be either stored frozen (–80 or –20°C) or loaded on a 12% sequencing gel.
11. Prerun the sequencing gel at a constant temperature of 50°C in 1X TBE. When the gel is at the appropriate temperature, heat the DNA and loading buffer mixture samples at 95°C for 5 min. Centrifuge and keep on ice until required. Wash the wells thoroughly to remove traces of urea that leached out during the prerun. Load the samples and run the gel at 50°C until the bromophenol blue dye has migrated ~30 cm from the wells. As a marker, a 5'-phosphorylated 24-mer platinated oligonucleotide (150 pg) is also loaded in one of the lanes (*see Note 21*).
12. Cut the nylon membrane (Hybond N+; *see Note 22*) and four pieces of Whatman 3MM to the size of the gel area to be transferred. Presoak the nylon membrane and one piece of Whatman 3MM paper in 10X TBE.
13. Place the glass plate horizontally on the bench with the gel face up. Cover the gel from the wells to the bromophenol blue dye front with the nylon membrane followed by one piece of Whatman 3MM paper (*see Note 23*).
14. Lay three more pieces of dry Whatman 3MM paper on top followed by a glass plate. Place a modest weight on top of the plate, e.g., two 500-mL bottles filled with water. Allow the DNA to transfer for 1.5–3 h (*see Note 24*).
15. Fix the DNA on the membrane by placing it (DNA side up) in 0.4 M NaOH for 20 min without submerging the membrane. The nylon membrane can be used

immediately (or it can be covered with Saran Wrap and kept in a cold room until required). Wash the nylon membrane in 5X SSC for 2 min.

16. Roll the nylon membrane in a nylon mesh and place in a hybridization bottle (*see Note 25*). Pour 15 mL of hybridization buffer into the bottle, and coat the membrane in buffer by rotating the bottle (*see Note 26*). Prehybridize at 42°C for 10 min. Add the radiolabeled probe to the bottle, and incubate in an oven at 42°C for 16 h. The solution containing the radiolabeled probe can be decanted into a suitable container and reused if necessary.
17. Carefully remove the membrane from the hybridization bottle with forceps. Wash the membrane twice for 10 min each time with 1 L of 1X SSC containing 0.1% SDS (warm to >30°C; *see Note 27*).
18. Cover the membrane in Saran Wrap, and expose the membrane to Kodak X-ray film at -80°C or phosphorimager screens (*see Note 28*). An example of the type of image formed on the X-ray film or phosphorimager is shown in **Fig. 4B**.

3.6. Analysis of NER DNA Products by an End-Labeling Method

1. Each sample in this assay requires one-fifth of the components that are used for the assay described in **Subheading 3.5**. Therefore, 10- μ L reaction mixtures can be used to analyze NER in cell extracts: combine 4.0 μ L of cell extract plus buffer containing 0.1 M KCl, 2.0 μ L of 5X repair reaction buffer, 0.2 μ L of CPK, 0.3 μ L of 1 M KCl, and 2.5 μ L of H₂O. Incubate the reaction mixtures in the absence of DNA for 5 min at 30°C.
2. Add 1.0 μ L of DNA substrate (50 ng). Incubate at 30°C for a further 25–30 min. At this point, the reaction mixtures can be stored frozen at -20°C.
3. To analyze the release of DNA containing the lesion, a 34-mer oligonucleotide is used (5'-pGGGGGAAGAGTGCACAGAAGAAGAGGCCTGGTCTGp-3' with a phosphate group on the 3' end to prevent priming). This oligonucleotide is complementary to the DNA fragment excised during NER (**Fig. 5A**) and the position of the major 3'-incision site (**ref. 3**) is such that the 34-mer oligonucleotide has a 5' overhang. This 5'-overhang (shown in bold letters in **Fig. 5A**) is used as a template by Sequenase to incorporate radiolabeled dCMP on the 3'-end of the excised fragments. In the case of Pt-GTG-DNA substrate, add 1.0 μ L of oligonucleotide (stock solution 6.0 μ g/mL) to the 10- μ L reaction mixture. It is recommended that the oligonucleotide is titrated to determine the right concentration for end-labeling.
4. Heat the tubes at 95°C for 5 min. Centrifuge the tubes to pull down any liquid that has evaporated and condensed on the side.
5. Allow the DNA to anneal by leaving the tubes at room temperature for 15 min.
6. Make up a Sequenase enzyme/[α -³²P] dCTP mixture such that each reaction contains 0.13 U of Sequenase enzyme and 2.0 μ Ci of [α -³²P] dCTP. These components are diluted in the Sequenase dilution buffer that is provided by the manufacturer. Add this mixture to the tubes, and incubate at 37°C for 3 min. Add

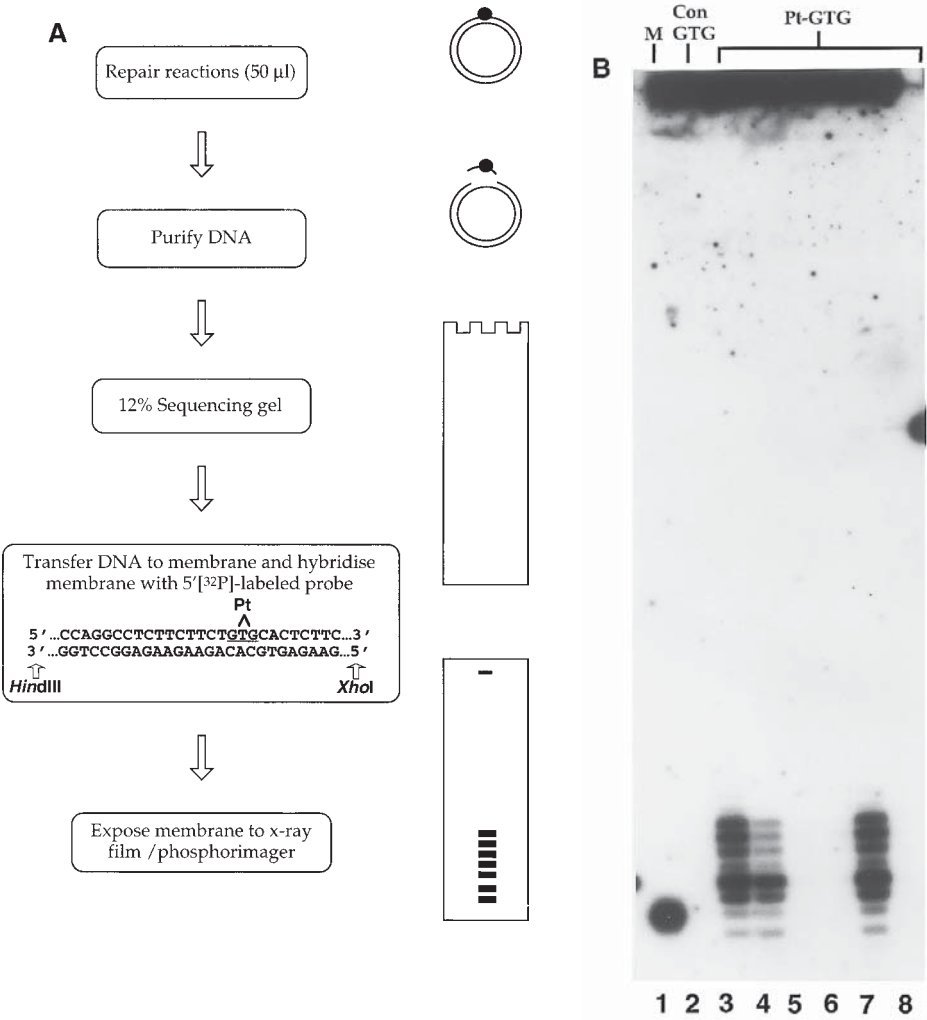


Fig. 4. Detection of excised fragments containing a 1,3-intrastrand d(GpTpG)-cisplatin adduct by Southern blot method. (A) Schematic. (B) Autoradiograph of the membrane containing the excised DNA fragments. Reaction mixtures (150 μ L) contained replication protein A (RPA)-depleted HeLa cell extract (144 μ g) and either recombinant wild-type (lanes 2, 3 and 7) or mutant (lanes 4–6) forms of human RPA. The reaction mixture without RPA is shown in lane 8. The reaction mixtures included DNA containing the lesion (lanes 3–8) or control DNA (without the lesion; lane 2). The samples were treated as described (schematic and **Subheading 3.5.**). Lane 1 contains a 5'-phosphorylated 24-mer oligonucleotide marker.

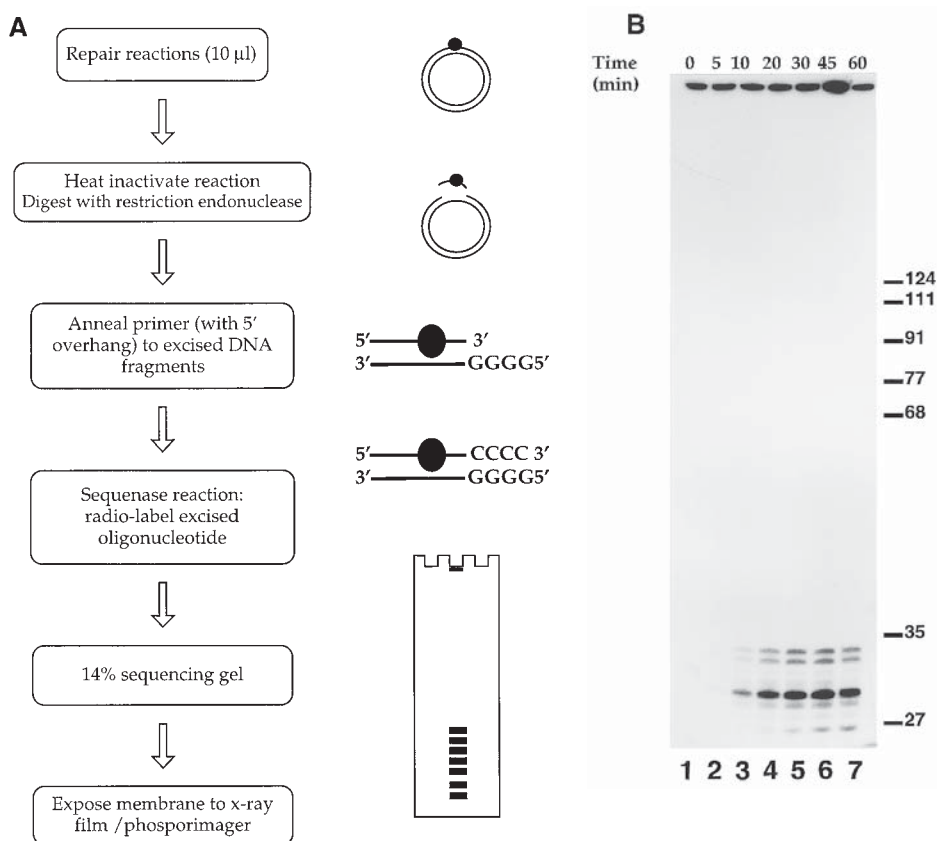


Fig. 5. Detection of excised fragments containing a 1,3-intrastrand d(GpTpG)-cisplatin adduct by the end-labeling method. (A) Schematic. (B) Autoradiograph of the dried sequencing gel containing the radiolabeled excised DNA fragments. A reaction mixture (80 μ L) contained RPA-depleted HeLa cell extract (100 μ g) and recombinant human RPA (1 μ g). This was incubated at 30°C for 5 min prior to adding DNA containing the 1,3-intrastrand d(GpTpG)-cisplatin crosslink. Aliquots of 10 μ L were removed at times 0, 5, 10, 20, 30, 45, and 60 min (lanes 1–7). The samples were treated as described (schematic and **Subheading 3.6.**). The positions of the radiolabeled *Msp*I-digested pBR322 DNA fragments are as shown (bold lines).

1.5 μ L dNTP mixture (10 μ M of each dATP, dGTP, TTP, and 5 μ M dCTP). Incubate at 37°C for a further 12 min.

7. Stop the reactions by adding 8.0 μ L of loading buffer. Mix thoroughly, and heat the tubes at 95°C for 5 min. Centrifuge the tubes, and keep them on ice.
8. Prerun a 14% sequencing gel until it reaches a temperature of 50°C (*see Note 29*). Thoroughly wash the wells prior to loading one-third of the sample (6–7 μ L)

in the wells. Include appropriate end-labeled markers (in this case pBR322 DNA digested with *Msp*I and end-labeled with [α - ^{32} P] dCTP; *see* **Note 30**). Electrophoresis should take place until the bromophenol blue dye migrates off the gel (which takes about 2 h).

9. Transfer the gel to 3MM paper, and dry the gel for 30–60 min. Expose the gel to Kodak BioMax film or phosphorimager screens (*see* **Note 28**). An example of the type of image formed on the X-ray film or phosphorimager is shown in **Fig. 5B**.

3.7. Analysis of NER Products

Using Internally Radiolabeled DNA Substrates

1. Set up the repair reactions (10 μ L) as described in **Subheading 3.6, step 1**.
2. Add 1.0 μ L of the internally radiolabeled DNA substrate (50 ng). Incubate at 30°C for a further 25–30 min. Stop the reactions by heating the reaction mixtures for ~3 min at 95°C and add 8.0 μ L of sequencing gel-loading buffer. At this point, the reaction mixtures may either be stored frozen at –20°C or kept on ice.
3. Prerun a 14% sequencing gel until it reaches a temperature of 50°C (*see* **Note 29**). Thoroughly wash the wells prior to loading most of the sample (~15 μ L) in the wells. Include appropriate end-labeled markers (in this case pBR322 DNA digested with *Msp*I and end-labeled with [α - ^{32} P] dCTP; *see* **Note 30**). Electrophoresis should take place until the bromophenol blue dye migrates off the gel (which takes about 2 h).
4. Transfer the gel to 3MM paper, and dry the gel for 30–60 min. Expose the gel to Kodak BioMax film or phosphorimager screens (*see* **Note 28**).

4. Notes

1. This is identical to 10X restriction endonuclease buffer (NEB buffer 2; New England Biolabs).
2. There are no dNTPs present in this buffer (cf. Chapter 29).
3. It is necessary to warm the solution to 37°C and stir for at least 1 h for complete dissolution of the yellow crystals. All cisplatin solutions should be protected from light.
4. Increasing the final concentration of oligonucleotide to 1.5 or 2.0 mM reduces the yield of platinated oligonucleotides, but gives a higher proportion of the oligonucleotide containing the desired 1,3-intrastrand crosslink relative to other platinated reaction products (*see* **Fig. 1**). This may be advantageous in obtaining a very pure preparation of platinated oligonucleotide.
5. This reduction in mobility results from the cisplatin-DNA adduct bending the DNA, adding a +2 charge, and increasing the mol-wt by 223 (17).
6. The dyes interfere with subsequent recovery of DNA.
7. TLC silica gel contains a UV chromophore that is masked by the DNA. The DNA appears as a shadow (19). Exposure to UV should be as brief as possible in order to avoid DNA damage.

8. This step determines the final purity of the platinated oligonucleotide, and thus, it is essential to be conservative in the amount of material excised from each band in order to avoid contamination from adjacent oligonucleotide species.
9. An alternative method for purification of the platinated oligonucleotide from preparative denaturing polyacrylamide gels avoids the use of any UV irradiation during visualization and excision of the desired reaction products. The platinated reaction products can be 5'-phosphorylated with T4 polynucleotide kinase and ATP prior to electrophoresis. ³²P-labeled oligonucleotide reaction products can be run in adjacent lanes to locate the desired 5'-phosphorylated platinated oligonucleotide products by autoradiography. The oligonucleotides excised from the gel must be dephosphorylated prior to any 5'-³²P-phosphorylation analysis for purity, and it is necessary to excise and analyze several gel fragments from each lane to ensure recovery of the desired species.
10. The identity of the 1,3-intrastrand d(GpTpG)-cisplatin crosslinks can be confirmed by enzymatic digestion of the platinated oligonucleotides to their component nucleosides using DNase I, P1 nuclease and alkaline phosphatase followed by reverse-phase HPLC analysis (K. J. Yarema and J. M. Essigmann, personal communication). It is generally easier, however, to confirm the presence of this lesion by restriction endonuclease or primer extension analysis after incorporation of the platinated oligonucleotide into closed-circular DNA (described in **Subheading 3.2.**). The platinum lesion can also be removed by treatment with sodium cyanide resulting in reversion of the oligonucleotide to the nonmodified form.
11. The 1,3-intrastrand d(GpTpG)-cisplatin crosslink is located within a unique *Apa*LI restriction site. Resistance to cleavage by this enzyme is diagnostic for the presence of the cisplatin-DNA adduct (**3,17**). Platinated (but not control) DNA preparations may be cleaved with *Apa*LI after completion of complementary strand synthesis to linearize any molecules lacking the 1,3-intrastrand cisplatin crosslink. This is conveniently done by supplementing the 200-μL reactions with 50 μL of 10X NEB restriction digestion buffer 4, 5 μL of 10 mg/mL BSA, 10 μL of *Apa*LI (10 U/μL), and H₂O to 500 μL and incubating for a further 3 h at 37°C; 2.5-μL aliquots can be analyzed as described in **step 3, Subheading 3.2.**
12. The presence of gapped circular DNA molecules (these have a mobility in between closed-circular and linear DNA forms under the electrophoresis conditions described) indicates that insufficient dNTPs or T4 DNA polymerase was present in the reaction.
13. The centrifugation conditions described give a good separation of closed-circular DNA from nicked-circular and linear forms
14. DNA containing CsCl may be dialyzed against TE buffer rather than desalted in a Centricon 100 ultrafiltration unit, but a subsequent concentration step may then be required.
15. In addition to resistance to cleavage by *Apa*LI (*see Note 11*), the presence of a site-specific cisplatin-DNA crosslink in DNA substrates can be confirmed by primer extension analysis of the damaged DNA strand (**3**).

16. The stock solution for the 24-mer oligonucleotide marker is made by adding 0.2 μL of the reaction mixture to 199.8 μL of TE. For a gel, use 1 μL of this plus 4 μL of TE and 4 μL of sequencing gel-loading buffer.
17. Sephacryl S-400 resin comes as a suspension in 20% ethanol. Spin columns (1 mL) are prepared by washing in TE six times (each 100- μL wash is centrifuged at 2000 rpm [320g] for 1 min. This step is very important in order to remove small DNA fragments produced by the T5 exonuclease digestion. These fragments inhibit the NER reaction.
18. The radiolabeled DNA needs to be used within 1 to 2 wk, because the substrate undergoes both radioactive decay and radiolytic decomposition.
19. Pipets with drawn-out tips are simply made by heating the tip of the Pasteur pipet in a gas burner flame and pulling the molten tip in a curve with a pair of forceps until the glass has a very fine diameter. The glass is snapped near where the glass begins to taper. The bore should be ~0.5–1.0 mm in diameter. When removing the alcohol, make sure that the tip of the drawn-out Pasteur pipet is facing away from the DNA pellet.
20. Digesting the DNA with *Hind*III and *Xho*I prior to gel electrophoresis allows detection of uncoupled incisions made either 3' (*Hind*III) or 5' (*Xho*I) to the lesion (20).
21. As an aid to identifying the platinated oligonucleotides formed during the dual-incision process with Pt-GTG DNA substrates, the 5'-phosphorylated 24-mer oligonucleotide prepared in **Subheading 3.1.** can be loaded alongside the products of the repair reaction.
22. Other types of membrane, e.g., Electran[®] positively charge nylon membrane (BDH), do not work as well with this procedure.
23. Remove any air bubbles between the membrane and the gel by rolling a glass pipet over the Whatman 3MM paper. The presence of air bubbles can interfere with the capillary transfer of DNA.
24. Always handle the nylon membrane by the edges.
25. Ensure that the leading edge formed by the membrane/mesh rotates into the buffer when placed in the hybridization oven.
26. SDS precipitates cause background spots and smears. It is important to avoid this. The solution can be stored at room temperature. If precipitates are seen, the solution should be filtered through 0.22- μm Nalgene filters. Warm the solution to 55°C before adding it to the hybridization bottle.
27. Keeping the wash buffer at >30°C prevents the SDS from precipitating out of solution. The stringency of wash depends on salt concentrations. Check for localized radioactivity after the second wash using a Geiger counter. If the membrane needs to be reprobed, soak the membrane in 0.4 M NaOH for 30 min. Wash in 5X SSC for 10 min, or boil the blot in 0.5% SDS.
28. The exposure time to X-ray film will depend on the type of film used. Kodak XOMAT AR is good for overnight exposures if the Geiger counter readings from the membrane (**Subheading 3.5.**) or dried gel (**Subheadings 3.6.** and **3.7.**) are in the range of ~10–50 counts/s. Alternatively, another type of X-ray film (Kodak BioMax MS) or a phosphorimager screen (Molecular Dynamics) is four times

more sensitive compared to Kodak XOMAT AR. In the case of the more sensitive alternatives, the exposure times are ~1–4 h where >50 counts/s are registered on the Geiger counter.

29. Using a 14% polyacrylamide gel allows separation of the 22–27 nucleotide fragments from the faster migrating salt front.
30. The DNA markers (*Msp*I-digested pBR322 plasmid) are end-labeled in Klenow buffer and [α - ^{32} P] dCTP with DNA polymerase I (Klenow fragment). The reaction is incubated on ice for 30 min. The reaction is terminated by the addition of 0.5 M EDTA, pH 8.0, to a final concentration of 20 mM. Loading ~1–2 ng of this marker on a sequencing gel gives an appropriate signal after a 4- to 5-h exposure to Kodak BioMax X-ray film. The marker is stable for several weeks even when repeatedly thawed and frozen at -20°C .

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In Vitro Chemiluminescence Assay to Measure Excision Repair in Cell Extracts

Bernard Salles and Christian Provot

1. Introduction

Nucleotide excision repair (NER) activity can be directly measured in whole-cell extracts by quantifying either the incorporation of radiolabeled deoxynucleotide during the repair synthesis step in damaged plasmid DNA (**1**; *see* Chapters 25–27, 29) or the excision of a previously labeled oligonucleotide containing a unique lesion (**2**; *see* Chapter 30). These two assays have been developed with cell-free extracts and more recently with purified proteins, leading to a deeper understanding of the proteins involved at each step of the NER reaction (**3,4**). Each assay has advantages and drawbacks. One advantage of the repair synthesis assay is that the substrate, damaged plasmid DNA, is relatively easy to prepare. However, the repair signal generated in the repair synthesis assay (i.e., radiolabel incorporation) may not reflect the incision activity of cell extracts where there are variations in length of the synthesized DNA fragment (**5**). Two methods designed to dissociate incision/excision from the repair synthesis step overcome this drawback: (1) the assay is performed with purified proteins involved only at the incision step (**6**), or (2) the repair synthesis step is blocked by addition of aphidicolin (a DNA polymerase inhibitor) and omission of dNTPs in the reaction mixture, leaving the incision activity unimpaired; the incised intermediates are subsequently purified and then labeled in a DNA polymerization reaction with the Klenow fragment of *Escherichia coli* DNA polymerase I (**7,8**; *see* also Chapter 30). Comparison between the repair synthesis yield and incision activity with cell extracts shows a direct correlation, indicating that the repair synthesis assay does permit measurement of incision activity in an NER reaction with cell-free extract (**9**).

However, the repair synthesis assay requires purification of damaged plasmid, use of radioactivity, and gel electrophoresis to separate repaired plasmid DNA from free, labeled dNTP. The latter step has been simplified by the use of a gel-filtration column in place of an agarose gel (10). In order to simplify the repair synthesis assay further and to increase its applicability, we have adapted the fluid-phase procedure to solid-phase (11). Plastic microplate wells coated with poly-L-lysine (Fig. 1, step 1) permit quantitative adsorption of plasmid DNA (damaged or otherwise) (step 2). Plasmid DNA can be damaged by the genotoxic agent either before (step 2) or after (step 3) adsorption. DNA damage is processed by repair enzymes allowing the incorporation of labeled deoxynucleotide during the repair synthesis step (step 4a). In the case of the incision reaction (7), preincised intermediates are formed and then labeled in a DNA polymerization step with Klenow fragment (step 4b). Incorporation of modified deoxynucleotide is visualized subsequently in an ELISA-like reaction (step 5) using chemiluminescent detection (step 6). The adsorption of DNA on a solid phase allows the replacement of all the separation procedures used in liquid-phase assays (agarose or sequencing gel) by a simple washing between each step. The chemiluminescent assay used for the detection of DNA damage, termed the 3D-assay (damaged DNA detection assay), can be readily automated (12) (see Note 1). All DNA damage is susceptible to detection, since whole-cell extract contains NER as well as the base excision repair proteins. Oxidative agents may thus be detected. Moreover, under conditions of controlled reactive oxidative species production, antioxidizing agents can be identified by inhibition of the repair signal. Alternatively, this assay is a convenient tool with which to analyze the excision repair activity in different cell extracts and can be used to screen repair inhibitors.

2. Materials

2.1. Sensitization of Microplate Wells

1. Microplates: 96-well Microlite™ 2 with Removawell® strips (Dynex Technologies, Chantilly, VA).
2. 5X PB: 50 mM phosphate buffer, pH 7.0.
3. 5X PBS: 50 mM phosphate buffer, 685 mM NaCl, pH 7.4.
4. PBST: 1X PBS, 0.1% Tween-20.
5. Poly-L-lysine hydrobromide (mol-wt range 15,000–30,000), 1 mg/mL stock solution. Store at –20°C.

2.2. Preparation of Plasmid DNA and Damaging Treatment

1. Qiagen columns.
2. TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
3. UV-C (254 nm) light and UV-C dosimeter.

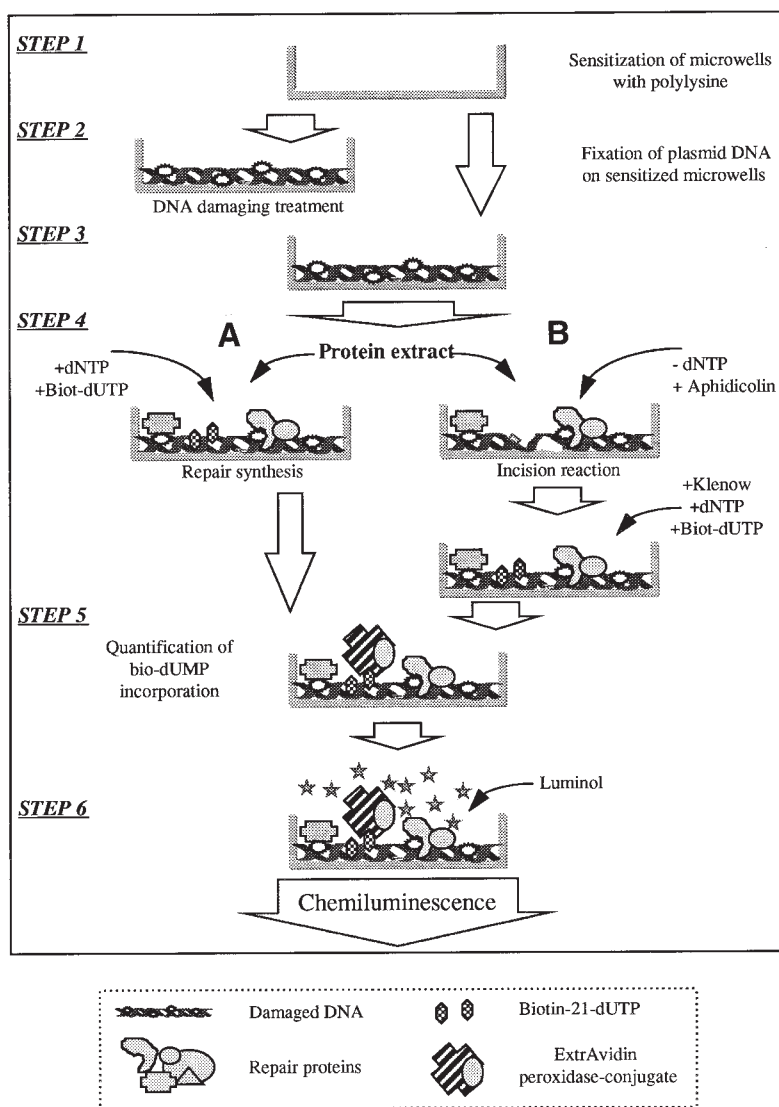


Fig. 1. Schematic representation of the 3D-assay. Plasmid DNA is adsorbed in sensitized microplate wells (either undamaged, **step 2**, or previously damaged, **step 3**). An excision repair reaction is performed with cell extracts (**step 4**). The repair synthesis incorporates or modified nucleotides (**step 4A**). In the case of an NER reaction, the repair synthesis step is blocked, and incised intermediates are used as substrates in a replication reaction with Klenow polymerase (**step 4B**). The extent of incorporation of modified nucleotides is determined in an ELISA-like reaction (**step 5**) with chemiluminescence detection (**step 6**).

2.3. DNA-Damaging Treatment in Microplate Wells

Drug is dissolved in water, 10 mM phosphate buffer, pH 7.2, or dimethylsulfoxide (DMSO; *see Note 2*).

2.4. Cell Extract Preparation

1. Cell lines are cultured either attached or in suspension (*see Note 3*).
2. Buffer A: 10 mM Tris-HCl, 10 mM MgCl₂, 10 mM KCl, pH 7.5. Store at -20°C .
3. Proteinase inhibitor stock solutions: Aprotinin (4 mg/mL in 10 mM HEPES, pH 8.0); chymostatin (3 mg/mL in DMSO); pepstatin (1.5 mg/mL in 70% ethanol); leupeptin (3 mg/mL in H₂O); phenylmethylsulfonyl fluoride (PMSF; 17.4 mg/mL in isopropanol). Store in aliquots at -20°C .
4. Buffer A* is prepared fresh on the day of the protein extract preparation:
 - a. 10 mL of buffer A.
 - b. 50 μL of 1 M dithiothreitol (DTT).
 - c. 5 μL each of chymostatin and leupeptin.
 - d. 10 μL each of aprotinin and pepstatin.
 - e. 50 μL of PMSF.
5. Saturated ammonium sulfate solution, pH 7.5.
6. Buffer B: 50 mM Tris-HCl, 10 mM EDTA, 100 mM KCl, 25% sucrose, 50% glycerol, pH 7.5. Store at -20°C .
7. Solid ammonium sulfate (finely ground with a pestle and mortar).
8. 1 M NaOH.
9. Dialysis buffer: 25 mM HEPES-KOH, 0.5 mM EDTA, 17% glycerol, 2 mM DTT, 100 mM KCl, pH 7.8.

2.5. DNA Repair Reaction

2.5.1. Repair Synthesis Reaction

1. 5X "RS" buffer: 220 mM HEPES-KOH, pH 7.8, 35 mM MgCl₂, 2.5 mM DTT, 2 μM each dGTP, dCTP, dATP, and biotin-21-dUTP (Clontech), 50 mM phosphocreatine, 250 $\mu\text{g/mL}$ creatine phosphokinase, 10 mM EGTA, 17% glycerol, 0.5 mg/mL bovine serum albumin (BSA). (*See Note 4.*) Store in aliquots at -70°C .
2. 3 M KCl.
3. 1 M ATP.
4. Cell extract stored frozen at -70°C .
5. Dialysis buffer (*see item 9, Subheading 2.4.*).
6. PBST (*see item 4, Subheading 2.1.*).

2.5.2. Incision Reaction

1. 5X "IR" buffer: 220 mM HEPES-KOH, pH 7.8, 25 mM MgCl₂, 45 μM aphidicolin, 2.5 mM DTT, 50 mM phosphocreatine, 250 $\mu\text{g/mL}$ creatine phosphokinase, 10 mM EGTA, 17% glycerol, 0.5 mg/mL BSA. Store in aliquots at -70°C .
2. **Items 2–6 of Subheading 2.5.1.**
3. Klenow fragment of *E. coli* DNA polymerase I.

4. Klenow buffer: 90 mM HEPES, pH 6.6, 5 mM MgCl₂, 0.4 μM each of dGTP, dCTP, dATP, and biotin-21-dUTP (Clontech).

2.6. Chemiluminescent Detection

1. ExtrAvidin peroxidase (Sigma).
2. Acetylated BSA (15 mg/mL).
3. IGEPAL CA-630 (Sigma).
4. Hydrogen peroxide solution, 30% (w/w).
5. 40X Enhanced luminol:
 - a. 75 mg Luminol (Aldrich).
 - b. 16 mg 4-iodophenol (Aldrich).
 - c. 2 mL of DMSO.
6. 100 mM Tris-HCl, pH 8.5.

3. Methods

All steps are performed in a 50-μL final volume/well.

3.1. Sensitization of Microplate Wells (Step 1)

1. Dilute poly-L-lysine stock solution in 1X PBS to 0.5 μg/mL final concentration.
2. Add 50 μL of diluted poly-L-lysine solution to each well.
3. Incubate overnight at 4°C without shaking (*see Note 5*).
4. Rinse three times with PBST.

3.2. Preparation of Plasmid DNA and UV-C Treatment

1. Purify plasmid DNA using a Qiagen column according to manufacturer's instructions (*see Note 6*).
2. UV-C damaging treatment is performed when the microplate assay is to be used to study NER (*see Note 7*).
 - a. Dispense 50-μL drops of plasmid DNA (50 μg/mL) in a Petri dish on ice.
 - b. UV-C irradiate the DNA at the desired dose, e.g., 300 J/m² (determined with a dosimeter).
 - c. Pool the drops, and store in aliquots at -70°C.

3.3. Plasmid DNA Adsorption (Step 2) and Damaging Treatment in the Microplate (Step 3)

1. Add plasmid DNA (1 μg/mL) in 1X PB to the microplate wells. When UV-C-damaged plasmid (from **Subheading 3.2.**) is used, omit **steps 4 and 5**. Be sure to include several wells with undamaged DNA as a control.
2. Shake at 30°C for 30 min.
3. Rinse twice with PBST.
4. Apply the DNA-damaging agent (drug or radiation) to the treatment wells. For chemical agents, incubate at 30°C for 30 min.
5. Rinse three times with PBST (*see Note 8*).

3.4. Cell Extract Preparation

The following method was adapted from Manley et al. (13) (see Note 9). All purification steps should be carried out at 4°C.

1. Measure the packed cell volume (PCV).
2. Add 4 PCV of buffer A* to the pellet in a beaker.
3. Keep on ice for 20 min.
4. Disrupt the cells by 10–20 strokes with a dounce homogenizer (B pestle).
5. Verify cell disruption by visual inspection under a microscope.
6. Slowly add 4 PCV of buffer B, followed by 1 PCV of saturated ammonium sulfate (pH 7.5) on a magnetic stirrer (~60 rpm).
7. Continue to stir for 30 min.
8. Centrifuge at 42,000 rpm in a SW 50 rotor (212,000g) for 3 h at 2°C.
9. Pipet the supernatant (normally 3–3.5 mL/5 mL of centrifuged solution) into a clean beaker.
10. Slowly add 0.33 g of solid ammonium sulfate per mL of solution.
11. Add 10 μ L of NaOH/g of ammonium sulfate added.
12. Stir for 30 min.
13. Centrifuge at 10,000g for 20 min.
14. Discard the supernatant, and resuspend the protein pellet in dialysis buffer (~300 μ L/1 mL PCV).
15. Dialyze overnight at 4°C
16. Determine the protein concentration (see Note 10).

3.5. DNA Repair (Step 4)

3.5.1. Repair Synthesis Assay (Step 4A)

1. Prepare the reaction mixture as follows (see Note 11):
 - a. 1X “RS” mix 200 μ L of 5X “RS”
 - b. 70 mM KCl x μ L (take into account the KCl in the protein extract)
 - c. 2 mM ATP 20 μ L of 1 M stock solution diluted 1:10
 - d. Extract to obtain 150 μ g/reaction
 - e. Water to 1 mL final volume (sufficient for 18–19 reactions).
2. Incubate at 30°C for the desired time without shaking (standard reaction time is 3 h).
3. Rinse three times with PBST.

3.5.2. Incision Assay (Step 4B)

1. Assemble the reaction as follows:
 - a. 1X “IR” mix 200 μ L of 5X “IR”
 - b. 70 mM KCl x μ L (take into account the KCl in the protein extract)
 - c. 2 mM ATP 20 μ L of 1 M stock solution diluted 1:10
 - d. Extract To obtain 150 μ g/reaction
 - e. Water To 1 mL final volume (sufficient for to 18–19 reactions).

2. Incubate at 30°C for the desired time (standard reaction time is 2 h).
3. Rinse three times with PBST.
4. Add 0.008–0.02 U/ μ L of Klenow fragment in Klenow buffer.
5. Incubate for 10 min at 30°C.
6. Rinse three times with PBST.

3.6. Detection (Steps 5 and 6)

1. Add ExtrAvidin peroxidase diluted 1:10,000 in 1X PBS containing 0.25 mg/mL acetylated BSA and 0.05% IGEPAL (*see Note 12*).
2. Incubate at 30°C for 30 min with shaking.
3. Rinse five times with PBST.
4. Protect the microplate from light, and add the visualization buffer containing:
 - a. 4 μ L of 40X enhanced luminol
 - b. 4 μ L of H₂O₂ diluted 1:100 in water
 - c. 100 mM Tris-HCl, pH 8.5, to a 1-mL final volume.
5. Incubate at 30°C for 5 min with shaking.
6. Measure the emitted light with a luminometer (expressed in relative light units [RLU]). To account for inherent variations in such an ELISA-like assay as well as deterioration in quality of some of the test components that might occur with time, repair activity is calculated as the ratio of RLU in treated vs untreated plasmid DNA.

4. Notes

1. The “3D” kit is available from SFRI, Berganton, St-Jean d’Illac, 33127, France.
2. Up to 20% DMSO final concentration does not alter the binding of plasmid DNA.
3. Frozen HeLa cell pellet can be obtained from Computer Cell Culture Cie (Mons, Belgium). Lymphoblastoid cell lines sometimes grow slowly with a high percentage of dead cells. Dead cells are responsible for increased radiolabel incorporation in untreated plasmid DNA (most probably owing to the presence of nucleases). When such cultures are used, cells should first be purified on a Ficoll gradient to eliminate dead cells. Attached cell lines are rinsed with cold PBS and detached in a small volume of PBS using a rubber policeman, then pooled, and centrifuged before preparation of the extract.
4. EGTA added to the reaction does not modify the extent of repair synthesis, but partially inhibits nonspecific nuclease activity when present in the cell extract preparation.
5. DNA adsorption can also be performed for 1 h at 37°C.
6. We have used highly purified DNA as reported in the liquid-phase method (*1*). From tests of more convenient plasmid DNA purification protocols, such as the Qiagen columns, we do not observe increased incorporation in the undamaged plasmid DNA. We therefore routinely use plasmid DNA prepared with the Qiagen columns.
7. In addition to the formation of pyrimidine dimers and 6-4 photoproducts, UV-C irradiation oxidizes DNA bases producing lesions that are recognized by the base

excision repair (BER) mechanism. The higher the UV dose, the higher the amount of oxidized nucleotides. To limit the participation of BER in the repair reaction, we irradiate pBS plasmid (~3 kb) with 300 J/m² of UV-C, which produces a low yield of incorporation with an NER-deficient xeroderma pigmentosum cell-free extract. Another possibility is to use cisplatin or acetoxy-acetyl-aminofluorene to induce DNA lesions that are only recognized by the NER process.

8. The amount of adsorbed DNA is unaffected by the presence of damage on DNA; 1 µg/mL of plasmid DNA corresponds to about 40 ng of adsorbed DNA in the microwell. Plasmid DNA concentration can be quantified with the fluorescent dye Picogreen (Molecular Probes, Eugene, OR) diluted 1:2500 in TE. Fluorescence is measured with a microplate fluorometer. Plasmid DNA desorption can be achieved by incubation with 1 M MgSO₄ at 30°C for 20 min.
9. We have tested various extraction procedures, and found they give more or less the same level of repair activity. However, in the case of rodent cell extracts, which exhibit a low level of repair activity, we use nuclear cell extracts (14).
10. Protein concentration is usually 20–30 mg/mL. Do not use cell extracts with <10 mg/mL protein, because low protein concentration necessitates the addition of a large extract volume, which causes partial inhibition of the repair reaction and incomplete repair activity.
11. The reaction mixture can be adapted to test the effect of protein or KCl concentration or any other parameter on the repair signal.
12. During the repair synthesis step, digoxigenylated-11-dUTP can be used instead of biotin-21-dUTP (at the same concentration). Consequently, the visualization procedure is performed with antidigoxigenin antibody conjugated with alkaline phosphatase diluted 1:10,000 in the same buffer (1X PBS containing 0.25 mg/mL acetylated BSA and 0.05% IGEPAL). Alkaline phosphatase activity is determined quantitatively by incubation of the complex with Lumi-Phos 530 (Lumigen Inc.) for 15 min and luminescence detection.

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Physical Monitoring of HO-Induced Homologous Recombination

Allyson Holmes and James E. Haber

1. Introduction

The repair of chromosomal double-strand breaks (DSBs) in *Saccharomyces cerevisiae* occurs most efficiently by homologous recombination. Homothallic mating-type (*MAT*) switching provides the most well-characterized system to study DSB repair by recombination in mitotic cells (1,2,3). *MAT* switching is a genetically programmed event in yeast haploid cells, initiated by the site-specific HO endonuclease (Fig. 1). This creates a DSB at *MAT* that can be repaired by homologous donor sequences, *HML* α or *HMR* α , located near the ends of the same chromosome. These donor loci are maintained in a silent chromatin structure that prevents both their transcription and cleavage by HO, though they can still serve as donors in recombination. Most of the time *MAT* α cells use *HML* α and thus switch to *MAT* α , whereas *MAT* α cells use *HMR* α to switch to *MAT* α . This change of mating type can be scored genetically and molecularly, since *Ya* and *Y α* sequences are different and have restriction endonuclease polymorphisms (Fig. 1).

In order to study the kinetics of this recombinational process, it is important to begin with a synchronous population of cells that have suffered a DSB at the same time. This is accomplished by placing the site-specific HO endonuclease gene under the control of a galactose-inducible promoter, and transforming this *GAL10::HO* construct into *ho* yeast cells, devoid of their endogenous G1-regulated HO endonuclease (4). To avoid glucose repression, cells are grown in lactate or raffinose prior to the addition of galactose to induce the expression of HO endonuclease. A 1-h induction is sufficient to ensure HO cleavage of its target site at the *MAT* locus, after which time cells are washed and reintroduced into glucose medium to repress further HO expression and to

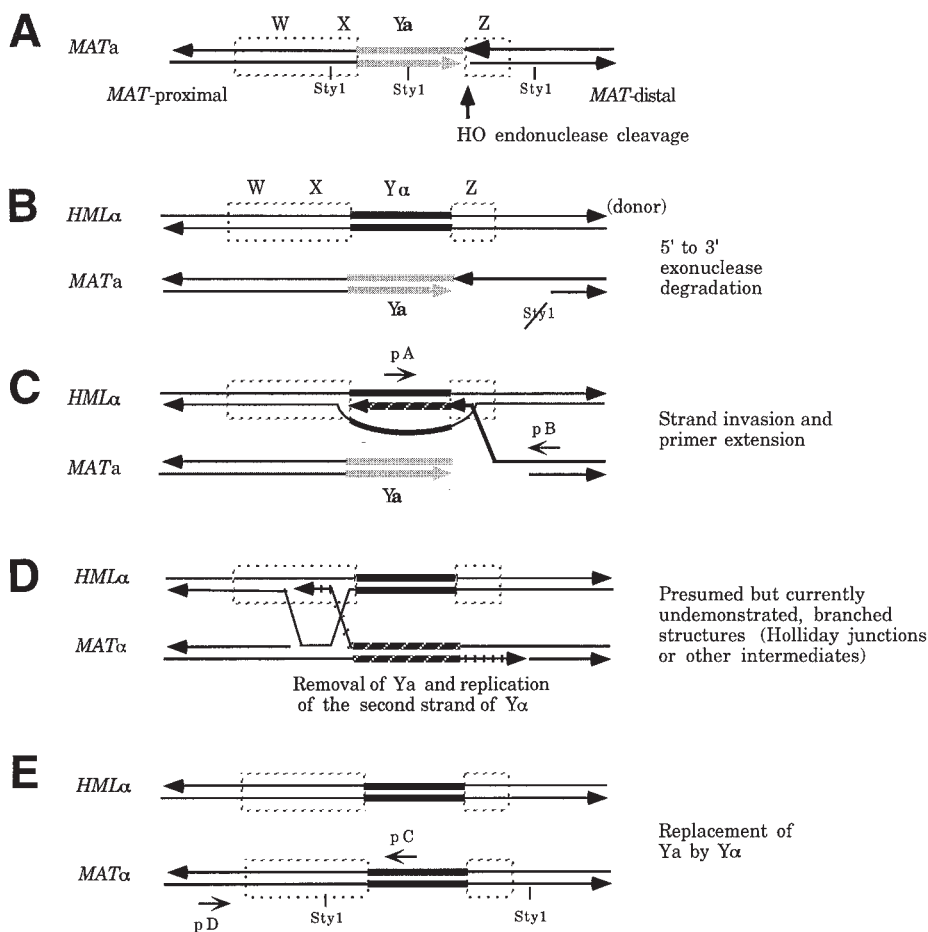


Fig. 1. Molecular model of mating type switching. A DSB is induced at the Y/Z junction by HO endonuclease. 5'–3' Exonucleolytic degradation creates a 3' single-stranded tail that invades the homologous silent donor sequence, *HML* α . Strand invasion and repair synthesis can be monitored using a unique set of primers (pB and pA), located distal to *MAT*, and within *HML* α . Final product formation can also be detected by PCR using *MAT*-proximal and *Y* α primers (pD and pC).

allow the completion of recombination. Cells are harvested at various intervals, and genomic DNA is isolated (5) to monitor the progress of recombination. This DNA is then analyzed by the polymerase chain reaction and Southern blots to detect different recombinational intermediates. These physical monitoring techniques are commonly used to identify the molecular intermediates and kinetics of *MAT* switching, as well as during other HO-induced repair events (Fig. 2).

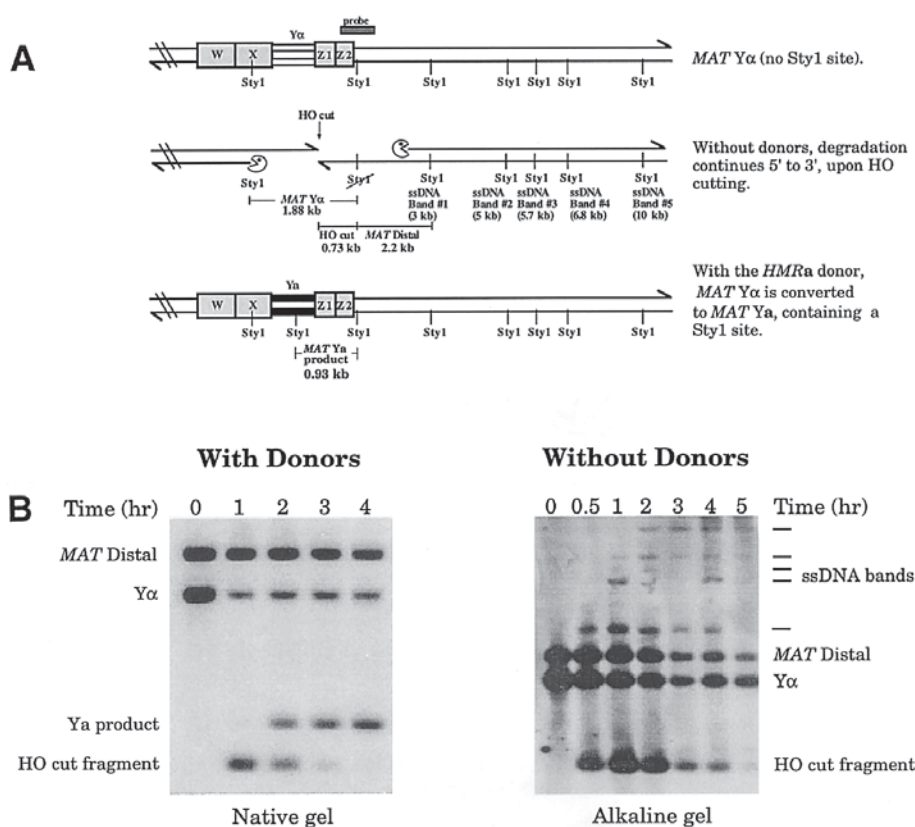


Fig. 2. (A) Schematic diagram showing the detection of 5'–3' degradation of HO-cleaved DNA. (B) Southern analysis of a *MAT* Y α strain, with and without donors. With donors, recombinant product is seen 1 hour after cutting by HO endonuclease. Without an available donor, *MAT* switching cannot occur, so 5'–3' degradation proceeds. This degradation is visualized on an alkaline gel as higher-mol-wt fragments.

The first intermediate detected is HO cleavage, seen as a smaller restriction fragment. This DSB is processed into a longer 3' single-stranded DNA (ssDNA) tail intermediate, which presumably occurs by the action of a 5'–3' exonuclease, and can be seen 15 min after induction (6). This 3' ssDNA tail invades the homologous donor sequence, and DNA synthesis is primed from the 3'-end. Strand invasion and repair synthesis are detected in 30 min upon DSB formation by a PCR assay, using primers that will only yield a PCR product if at least part of the donor locus is copied, so that the primer-extended, invading *MAT* strand provides a template for both primers. Another set of primers may be used to detect the final recombinational product, which is observed 1 h after

the DSB is made, coincident with the appearance of the product on Southern blots. Presumably there are other branched structural intermediates formed, such as Holliday junctions, nonhomologous ssDNA tails, and replication intermediates, but these have yet to be physically identified.

The protocols presented here describe the use of alkaline denaturing Southern blots, ssDNA dot-blot assays, and PCR to monitor the early steps of recombination after HO cleavage. Alkaline denaturing gels are used to detect the formation of long 3'-ended ssDNA tails, caused by the action of one or more 5'-3' exonucleases. As 5'-3' degradation proceeds, one or more restriction sites become single-stranded and cannot be cleaved. Thus, restriction endonuclease digestion yields higher mol-wt fragments that appear as partially digested DNA fragments, because they are partially single-stranded (**Fig. 2**). Since these fragments are partially (and variably) single-stranded, they do not produce discrete bands using nondenaturing gel electrophoresis (7). However, they can be seen using denaturing gels, so that the 3'-ended strand, which is not significantly degraded by 3'-5' exonucleases (8) is seen as a discrete band. The opposite strand, which is partially and variably degraded, is not seen. The extent of 5'-3' degradation depends on whether recombination can be completed. If donors are available, then degradation occurs at least several hundred base pairs distal to *MAT*, at about 1–2 nucleotides/s (7). However, when recombination is prevented in strains lacking donors or in strains deleted for various genes necessary for recombination, 5'-3' degradation can be detected at least 20 kbp distal to *MAT* using this method. Higher-mol-wt, partially single-stranded fragments cannot efficiently migrate through the gel, and thus are not seen. Southern blots of such denaturing gels can then be analyzed by phosphorimager to determine the extent, quantity, and kinetics of appearance of ssDNA. Alkaline-gel electrophoresis is an easy and informative way to analyze time-course DNA samples extracted from various genetic mutant backgrounds to examine the in vivo importance of specific proteins. Thus, this method can be used to determine the protein(s) involved in exonucleolytic activity, in regulating this degradation, and in the maintenance of HO-induced DSB ends. For example, deletions of both the *RAD50* and *XRS2* genes retard both 5'-3' degradation and the appearance of final products (9).

A more quantitative assessment of the amount of ssDNA can be obtained by using a nondenaturing dot-blot assay (7,10,11). Here, native genomic DNA is directly applied to a nylon membrane without denaturation and then hybridized with single-stranded probes to detect DNA that has become single-stranded. This method is more quantitative and allows detection of ssDNA, independent of its length. Although the dot-blot analysis is a more accurate method to quantitate ssDNA, it cannot be used to visualize the entire spectrum of intermediate events observed on alkaline gels, such as DSB formation, ssDNA intermediates, and final recombinant product formation.

Detection of subsequent steps, such as the initiation of new DNA synthesis after strand invasion, can be followed by a PCR-based assay. Using oligonucleotide primers that hybridize to unique sequences near *MAT* and within the donor, one can detect the strand invasion and extension intermediate (primers pA and pB) as well as the final recombination product with another set of primers (pC and pD; **Fig. 1**). If extension by DNA synthesis does not occur, then this intermediate is not seen, as in mutant strains of specific recombination-repair genes (*rad51*, *rad52*, *rad54*, and *rad57*), or in certain mutants defective in DNA replication (**12**; A. Holmes and J. E. Haber, unpublished).

Physical monitoring of HO-induced recombination is not limited to *MAT* switching, and can be used to study many interesting recombinational processes in yeast and in other organisms (**13–30**). The insertion of a synthetic HO endonuclease recognition site (commonly a 33- or 117-bp segment) at other genomic locations can be used to investigate aspects of both gene conversion and alternative homologous DNA repair events such as single-strand annealing (**31**). HO-induced DSBs can also be used to study nonhomologous end-joining repair (**25,32**), the *de novo* formation of new telomeres (**33,34**), recombination by HO-initiated meiotic DSBs (**35**), and novel means of acquiring a new chromosome end (**36**).

2. Materials

2.1. Induction of *MAT* Switching and Extraction of DNA

1. Selective medium (per 1 L): Dissolve into water 6.7 g/L yeast nitrogen base without amino acids (Difco), 20 g/L dextrose (Fisher Scientific), 20 g/L agar (Acumedia), 0.87 g/L amino acid (Sigma)/base mixture, missing one of the amino acids or bases being selected for (0.8 g adenine, 0.8 g arginine, 4 g aspartic acid, 0.8 g histidine, 2.4 g leucine, 1.2 g lysine, 0.8 g methionine, 2 g phenylalanine, 8 g threonine, 0.8 g tryptophan, 1.2 g tyrosine, and 0.8 g uracil; add all but one of the ingredients to make the desired selective mixture). Autoclave.
2. YP-lactate medium: 3.0% lactic acid (Fisher Scientific), 1.0% yeast extract (Difco), 2.0% peptone (Difco), neutralize to pH 5.5 using NaOH (Fisher Scientific).
3. 20% Galactose (Sigma): dissolve galactose at room temperature in water, and filter sterilize (do not autoclave).
4. 20% Dextrose (autoclave) (Fisher Scientific).
5. Acid- and base-washed glass beads, 0.5 μ m diameter (Sigma).
6. DNA extraction buffer: 100 mM Tris-HCl (Sigma), pH 8.0, 50 mM EDTA (Fisher Scientific), 2.0% sodium dodecyl sulfate (SDS, Sigma).
7. Equilibrated phenol, pH 8.0 (American Bioanalytical).
8. 3 M Sodium acetate, pH 5.2.
9. Isopropanol.
10. Ribonuclease A (Sigma).
11. TE: 10 mM Tris-HCl (Sigma), pH 7.5, 1 mM EDTA.

12. 70% Ethanol.
13. *Sst*I restriction enzyme with 10X reaction buffer.

2.2. Preparation of Alkaline Gel and Electrophoresis Conditions

1. Alkaline gel: 30 mM NaCl, 2 mM EDTA, agarose, water.
2. 1X Alkaline buffer: 30 mM NaOH, 2 mM EDTA, water (make fresh).
3. 6X Alkaline-loading buffer: 0.3 M NaOH, 6 mM EDTA, 18% Ficoll (type 400), 0.15% bromocresol green (Sigma), 0.25% xylene cyanole FF (store 4°C).
4. 3 M Sodium acetate, pH 5.2.
5. 0.5 M EDTA, pH 8.0.
6. 70% and 100 Ethanol.
7. TAE or TBE buffer with ethidium bromide (0.5 µg/mL):
TAE (Tris-acetate) 1X buffer: 0.04 M Tris-acetate, 1 mM EDTA.
TBE (Tris-borate) 1X buffer: 0.09 M Tris-borate, and 2 mM EDTA.

2.3. Single-strand DNA Dot-Blot Assay

1. Genomic DNA.
2. Gel-loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 15% (Ficoll-type 400) in water.
3. 20 and 10X SSC buffer: 20X is 3 M NaCl (175 g/L), 0.3 M Na₃citrate (88 g/L). Adjust pH to 7.0 with 1 M HCl.
4. 0.4 N NaOH.
5. 3 M sodium acetate, pH 5.0–6.0.
6. Microtiter dishes.
7. Nylon membrane (positively charged or neutral).
8. Minifold II slot-blot system (Schleicher and Schuell).

2.4. PCR of MAT Intermediates

1. 0.5-mL thin-walled tubes (USA/Scientific Plastics®).
2. PCR master mix (per 50-µL reaction):
5 µL 10X Thermophilic buffer (Promega)
5 µL 10X 25 mM MgCl₂
1 µL of 10 mg/mL bovine serum albumin (BSA)
1 µL of 10 mM dNTPs mix
Oligonucleotide primers (0.5 pmol/mL final concentration):
pA primer (*HML*): pGCAGCACGGAATATGGGACT
pB primer (*MAT* distal): pATGTGAACCGCATGGGCAGT
pC primer (*HML*): pAGATGAGTTTAAATCCAGCA
pD primer (*MAT* proximal): pTGTGTCTCACTATCTTGCC
5 ng of yeast genomic DNA
1 µL *Taq* polymerase (5 U/µL).
3. Paraffin oil.
4. PCR conditions: 25 cycles of 94°C for 1.5 min, 55°C for 2 min, 72°C for 3 min followed by a final extension step of 72°C for 7 min (PTC-100™ Programmable Thermal Controller, from MJ Research Inc.).

3. Methods

3.1. Induction of MAT Switching and Extraction of Time Course DNA (see Note 1)

1. Select a single yeast colony for growth overnight in 5 mL of glucose-containing medium at 30°C. If the galactose-inducible *GAL10::HO* gene is carried on a centromeric plasmid, use selective synthetic medium to retain the plasmid (37). This is unnecessary with a stably integrated *GAL10::HO* sequence (34).
2. Spin down the cells, and discard the supernatant. Resuspend the pellet in YP-lactate, and inoculate YP-lactate medium to give approx 10^7 cells/mL the next morning. Make sure the cells are shaking at 30°C with proper aeration (i.e., 0.5–1.0 L of culture/4-L flask).
3. When the appropriate cell density is reached, remove a 50-mL aliquot from the flask. The first aliquot or time-point will represent the uninduced (0-h) sample. Add 1/10 vol of 20% galactose to the remaining culture. For *MAT* switching assays, 30–60 min are enough time to induce cutting in most of the cells. After this time, remove another 50-mL aliquot, and add 1/10 vol of 20% glucose to stop cutting by HO endonuclease. Because the switched product can be recleaved by HO, it is necessary to stop HO expression. However, for strains that do not retain the HO cut site after recombining or that do not contain donors (Fig. 2), glucose addition is not necessary. Continue taking 50-mL aliquots as desired.
4. Centrifuge the samples, discard the supernatant, and resuspend the cells in 400 μ L of extraction buffer. Transfer the cells to a 1.5-mL Eppendorf tube, containing 400 μ L of phenol and 500 μ L of glass beads. The glass bead/phenol mixture can be prepared in advance and stored at 4°C in the dark.
5. Vortex the tube vigorously for 1–2 min, with occasional inversion of the tube to keep the beads well mixed. If the samples are not vortexed enough, the cell walls will not be broken efficiently, and the DNA yield will be low. Too much vortexing will result in shearing of the DNA. The samples can be stored on ice until the other time-course samples are ready (up to 6 h are fine). Add enough extraction buffer to the samples to equalize the volumes before centrifuging.
6. Centrifuge for 10–15 min at 4°C in a microcentrifuge.
7. Carefully remove the top aqueous layer, and transfer to a new microcentrifuge tube. To this new tube add 400 μ L of phenol, invert, leave on ice for 1–2 min, centrifuge 10–15 min as in step 6, and carefully extract the aqueous layer again. It is very important not to carry over protein from the organic/aqueous interphase.
8. Add 50 μ L of 3 M sodium acetate, pH 5.2, and 600 μ L of isopropanol. Mix.
9. Centrifuge the samples for 1 min at 4°C, and discard the supernatant.
10. Add 300 μ L of TE containing 10 μ g of RNase A (see Note 2). Incubate at 37°C for 30–60 min with occasional vortexing until the pellet has dissolved.
11. Add 30 μ L of 3 M sodium acetate and 300 μ L of isopropanol. Spin for 1–5 min at 4°C, and discard the supernatant. Rinse with 70% ethanol, dry the pellet, and resuspend in water or TE.
12. Digest the DNA with *StyI* restriction enzyme (see Note 3).
13. Use 25–50% of the total DNA to run on the alkaline denaturing gel.

3.2. Preparation of Alkaline Gel and Electrophoresis Conditions

This denaturing gel protocol is according to McDonnell et al. (38) and has been modified by Maniatis et al. (39).

1. Melt the agarose in 30 mM NaCl, 2 mM EDTA solution, pour into the electrophoresis tray, and let solidify. Once solidified, allow the gel to equilibrate 30 min or longer in alkaline buffer (*see Note 4*).
2. While the gel is equilibrating, precipitate the restricted DNA samples in 0.3 M sodium acetate, 5 mM EDTA, and 2 vol of ethanol. The EDTA is added to chelate Mg^{2+} so that the DNA will not precipitate in the alkaline buffer during electrophoresis. The addition of EDTA to the gel also prevents this.
3. Precipitate the DNA samples on dry ice for about 10 min, and centrifuge for 10 min. Discard the supernatant, wash the pellet with 70% ethanol, and dry.
4. Resuspend the pellet in 10–30 μ L of 6X alkaline gel-loading buffer. Denature the size markers by diluting a small volume of DNA into the loading buffer.
5. Load the DNA onto the equilibrated gel, and cover the gel with a glass plate to keep the gel in place (gels can detach from the base plate in alkaline buffer), and to prevent the bromocresol green dye from diffusing out of the gel, which happens at high pH. The use of 6X, concentrated, loading buffer also helps to visualize the length the DNA has run on the gel.
6. Once the DNA has migrated into the gel, carefully remove as much of the alkaline running buffer as possible with a pipet, leaving approx 1 mm covering the gel. Electrophoresis should be carried out at low voltages, since alkaline gels are not buffered and thus draw more current and heat up. Run at approx 1 V/cm, 20–24 h for a 0.8–1.2% gel of about 20 cm (*see Note 5*). Also, recirculate the buffer to prevent the anode from becoming too alkaline, and the cathode too acidic.
7. Once the dye has migrated halfway to two-thirds through the gel, stain the gel with 0.5 μ g/mL ethidium bromide in 1X TAE or 1X TBE buffer. Because ethidium bromide binds ssDNA very poorly, stain the gel for 30–45 min, and destain for another 30 min in TAE or TBE to visualize the bands more clearly with UV light.
8. Transfer the DNA to a positively charged nylon membrane, and hybridize according to Church and Gilbert (40), with double-stranded DNA (dsDNA) probes prepared by the random primer method (41).

3.3. ssDNA Dot-Blot Assay

The ssDNA dot-blot assay can be performed in conjunction with the alkaline gel method from the same time-course DNA to give a more precise determination of ssDNA in the sample, as demonstrated by Sugawara and Haber (7). For this method, genomic DNA from the time-course is blotted onto a nylon membrane, without denaturation, such that ssDNA, but not dsDNA in the sample will bind. The membrane is then hybridized with RNA probes, which bind preferentially to the ssDNA, increasing the specificity even more.

Theoretically, only ssDNA generated after HO cutting by exonuclease or helicase activity should appear, and no signal should be detected in the 0-h time-point. As a positive control, the same genomic DNA is denatured (becomes ssDNA), and should give a signal throughout the time-course (*see Note 6*).

1. Native samples (*see Note 7*):
 - a. Dilute 1 μg of genomic DNA into 200 μL of water (enough for two blots).
 - b. Add 100 μL of diluted DNA to a microtiter dish well containing 100 μL of SSC/dye mixture (5 μL of loading dye/10 mL of 20X SSC).
2. Denatured samples (positive control):
 - a. Dilute 1 μg of genomic DNA 1000-fold in water.
 - b. Aliquot 60 μL of 0.4 *N* NaOH into microtiter dish well.
 - c. Add 50 μL of the diluted DNA to the well. Mix.
 - d. Neutralize with 50 μL of 3 *M* sodium acetate/dye mixture (10 μL of loading dye/10 mL of sodium acetate).
3. Cut the nylon membrane to fit the minifold slot-blot apparatus, and assemble the dot-blotter according to the manufacturer's conditions.
4. Connect the hose of the slot-blotter to a vacuum, begin suction, and then add the samples to the wells of the apparatus.
5. Rinse each well with 500 μL of 10X SSC (still under suction).
6. Disassemble the slot-blotter, remove the nylon filter, and crosslink the DNA to the membrane with UV light.
7. Hybridize according to Church and Gilbert at 72°C (**40**), with RNA strand-specific probes (**42**). These RNA probes are easily made using an in vitro transcription kit from Promega (Madison, WI). An RNA probe hybridized to the nondegraded strand will give an increasing signal over time, using the native time-course DNA samples, if ssDNA is formed on HO cutting. The same probe will bind to the denatured DNA samples throughout the time-course. For quantitation purposes, normalize the signal in the native samples to the uninduced (0-h) denatured time-point DNA.

3.4. PCR of MAT Intermediates

1. Prepare enough PCR master mix of all components of the reaction, except the time-course DNA, to avoid variations.
2. Aliquot 5 ng of each DNA sample into 0.5-mL microcentrifuge tubes, and then add the same amount of master mix to each tube (*see Note 8*).
3. Overlay with 25–50 μL of paraffin oil, and place tubes in the PCR apparatus, using the conditions described in **Subheading 2.4**.
4. Electrophorese samples on neutral agarose gels.

4. Notes

1. The time-course induction protocol must be carried out with exponentially growing cells, since cells reaching stationary phase do not induce HO very well after galactose addition, and reliable kinetics of recombinational intermediates will be

- impossible. However, if the cell density is too low, it will be difficult to extract enough DNA to visualize on an alkaline gel.
2. The amount of RNase used in this step may seem excessive, but it is especially important to remove as much RNA from the sample as possible when performing the dot-blot assay (**Subheading 3.3.**), since RNA will give a high background and make quantitation impossible.
 3. It is very important to restrict completely the genomic DNA used for alkaline gels, since these partially digested fragments will make quantitation impossible, and will obscure the ssDNA. As shown in **Fig. 2**, there should be no higher mol-wt DNA in the uninduced (0-h) time-point.
 4. Adding NaOH to hot agarose will cause hydrolysis of the polysaccharides in the gel. Because ethidium bromide does not bind to DNA at high pH or to ssDNA very well, it is not added to the gel or alkaline buffer.
 5. Although the alkaline gel should be run slowly, it is not recommended to electrophorese much slower than 1 V/cm, since the bands start to become diffuse.
 6. One can also use denatured genomic DNA lacking hybridizable sequences as a negative control.
 7. The amount of DNA used for the native samples described in **Subheading 3.3.** is much higher than for the denatured samples since the amount of ssDNA produced after HO induction is very small. Therefore, we use more native DNA to generate detectable signals.
 8. Do not use more than about 5 ng of time-course DNA for the PCR assay (1% of the total time-course DNA), since the DNA contains impurities that can inhibit the PCR. Furthermore, only 25 cycles are used for this reaction, so that the amount of product is kept within a linear range, using the conditions described. The quality of the PCR may vary with different equipment, so the number of cycles needed to reach this linear range may vary slightly.

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Use of P Element Transposons to Study DNA Double-Strand Break Repair in *Drosophila melanogaster*

Daryl S. Henderson

1. Introduction

The combination of *rad* mutations and regulated HO endonuclease has proven to be a potent system for elucidating DNA double-strand break (DSB) repair mechanisms in *Saccharomyces cerevisiae* (see Chapter 32). An analogous system comprising mutagen-sensitive mutations (see Chapter 3) and P element transposons, whose transposition via a “cut and paste” mechanism induces a DSB at the site of excision, is being exploited in *Drosophila melanogaster* for the purpose of analyzing DSB repair in a multicellular organism. The full-length or “complete” P element is 2907 bp in length and has 31-bp inverted repeat termini (**1**) (**Fig. 1**). Functionally, it is a single gene consisting of four exons, numbered 0–3. In the germline, these are spliced together to produce an ~2.5-kb transcript specifying an 87-kDa transposase required for transposition (**2,3**). In somatic cells, a 97-kDa host-encoded protein binds at a site near the 3'-end of exon 2, preventing its splicing to exon 3 (**4,5**). This alternative transcript specifies a truncated polypeptide of 66-kDa that acts as a repressor of P element mobility (**6**). An in vitro-modified P element, $\Delta 2-3$, in which the intron between exons 2 and 3 has been precisely deleted, expresses a transposase that is active in all tissues (**2**). Several stably integrated derivatives of $\Delta 2-3$ have been isolated, which express high levels of transposase without undergoing mobilization themselves (**7**).

Transposase is a site-specific endonuclease that binds at sequences near both P element termini (**8**) and generates at each end a staggered cut with a 17-bp 3'-overhang (**9**). Efficient cleavage requires the presence of both 5'- and 3'-termini, suggesting that the two ends of the P element must physically interact

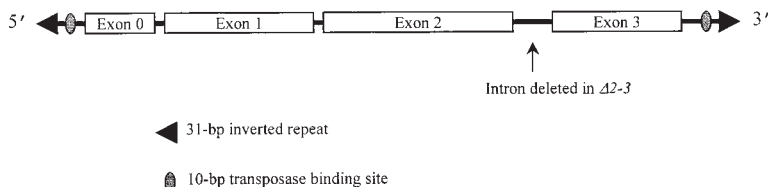


Fig. 1. Anatomy of a full-length P element. (Not drawn precisely to scale.)

prior to cutting. There is also evidence that a protein complex remains stably bound to these ends following cleavage, thereby protecting them from exonucleolytic degradation (9). On excision of the P element, host-encoded functions are required to repair the resulting DSB in cellular DNA. This was shown first by Banga et al. (10), who found that mutants of two repair genes, *mei-41* and *mus302*, experienced high levels of lethality when P elements in those strains were mobilized by transposase. *mei-41* is now known to be a homolog of the human genes *ATM* and *ATR* (11–13), all of which encode phosphatidylinositol kinase-like proteins involved in DNA damage checkpoints. Based on their findings, Banga et al. (10) proposed that P transposition could be used to identify DSB repair-defective mutants. Using that approach, two additional genes have since been shown to be required for DSB repair: *mus309* (14) and *mus209* (15). *mus309* encodes a homolog of the 70-kDa subunit of the mammalian heterodimeric protein Ku involved in DSB repair (14). Interestingly, the *mus309* product was originally identified biochemically as a factor that binds to P element sequences within the 31-bp inverted terminal repeats and was therefore named inverted repeat binding protein (IRBP) (16,17). *mus209* encodes a homolog of the DNA polymerase processivity factor proliferating cell nuclear antigen (PCNA) (18). Transposase-induced DSBs can be repaired by direct end-joining (19,20) and by gene conversion-like mechanisms (see Chapter 34). However, the precise functions of MEI-41, IRBP, and PCNA in DSB repair are unclear.

In principle, any P element insert might serve as a target sequence for transposase in this assay. However, different insertions undergo excision with different efficiencies. The minimal sequence requirements for transposition are 150 bp from both the 5'- and 3'-ends (21). P elements mutated in a transposase binding site or 31-bp inverted repeat are mobilized infrequently or not at all (21). Other factors that may affect excision frequency are the local chromatin environment and proximity of one P element to another. The *snw* mutant allele is an especially good substrate for transposase. It is actually a double-insertion mutation consisting of two internally deleted P elements oriented in a head-to-head fashion (22). Since neither element encodes a functional transposase, the

mutant is normally stable. However, when crossed to a transposase-expressing line, such as $\Delta 2-3$, one or the other P element in *snw* is excised very efficiently.

This chapter describes how to use the P element transposition system outlined above to identify *Drosophila* mutants deficient in DSB repair. **Subheading 3.1.** describes the genetic crosses required to set up the assay, using $\Delta 2-3$ as a source of transposase, *snw* as a target site, and the PCNA mutant *mus209B1* as an example of a repair-deficient line. **Subheading 3.2.** presents a simple method for cytological analysis of site-specific breakage of mitotic chromosomes. Details for analyzing *Drosophila* DSB repair at the molecular level can be found in Chapter 34.

2. Materials

2.1. Genetic analysis

1. Fly stocks:
 - a. Transposase source: $\Delta 2-3(99B)$ or $\Delta 2-3(68C)$ (see **Note 1**).
 - b. Transposase target: *snw* (see **Note 2**).
 - c. Mutant to be tested for DSB repair defect: e.g., *mus209B1* (15,18).
2. Basic equipment for *Drosophila* culture, e.g., vials, bottles, anesthetizers, dissecting microscope, and so forth, is required.

2.2. Cytological Analysis

1. Dissecting solution: 0.7% NaCl.
2. Forceps: (Dumont No. 5 or 5a).
3. Fixatives: 45 and 60% acetic acid.
4. Acetoorcein stains: 3% orcein in each of 45 and 60% acetic acid. Add 3 g of Gurr synthetic orcein (BDH, Toronto, Canada) to 100 mL of 45% or 60% acetic acid and boil with refluxing for 30 min. Filter through filter paper (e.g., Whatman no. 1), and store as a stock solution at 4°C in bottles sealed with parafilm. For current use, keep an aliquot of each stain in a 1.5-mL Eppendorf tube. Centrifuge at 12–14,000g for 10 min prior to use.
5. Tissue paper (e.g., Kimwipes).
6. 3MM paper for blotting.
7. Microscope slides and siliconized glass cover slips (24 × 24 mm²).
8. A flat ~1.5-cm-thick piece of hardwood approx. 15 × 10 cm² (optional).
9. Phase-contrast microscope with 60 to 100× objective lenses.

3. Methods

3.1. Generating Site-Specific DSBs

The exact way in which these crosses are done will depend on several factors, including the chromosomal location of the repair mutant of interest, whether it is fertile as a homozygote, and so forth. Mating schemes designed to test the DSB repair proficiency of mutagen-sensitive (*mus*) mutants on each of the X, 2nd, and 3rd chromosomes are described in ref. (10) and are similar to

Table 1
Crosses Used to Test for Defective DSB Repair in *mus209B1* Mutants

Genotype of progeny	No. of progeny	Relative viability
Cross 1 <i>snw/snw; mus209B1/CyO</i> × <i>+/Y; mus209B1/mus209B1; Δ2-3/Δ2-3</i>		
a <i>snw/Y; mus209B1/mus209B1; Δ2-3/+</i>	0	0.0
b <i>snw/Y; mus209B1/CyO; Δ2-3/+</i>	72	
c <i>snw/+; mus209B1/mus209B1; Δ2-3/+</i>	0	0.0
d <i>snw/Y; mus209B1/CyO; Δ2-3/+</i>	216	
Cross 2 <i>+/+; mus209B1/CyO; Δ2-3</i> × <i>snw/Y; mus209B1/CyO</i>		
e <i>+/Y; mus209B1/mus209B1; Δ2-3/+</i>	99	1.04
f <i>+/Y; mus209B1/CyO; Δ2-3/+</i>	191	
g <i>snw/+; mus209B1/mus209B1; Δ2-3/+</i>	0	0.0
h <i>snw/Y; mus209B1/CyO; Δ2-3/+</i>	228	

*a*The balancer chromosome *CyO* carries the dominant marker *Cy* and a wild-type allele of *mus209*. “Relative viability” is the ratio of *mus209B1* homozygotes (non-*Cy*) to their *mus209B1/CyO* (*Cy*) same-sex siblings in each cross. The values obtained for Cross 2 were multiplied by 2 since both parents were *mus209B1/CyO* heterozygotes and therefore expected to produce half as many *mus209B1* homozygous as heterozygous offspring (the *CyO/CyO* genotype is lethal).

those outlined here. As an example of this method, crosses that were used to demonstrate a requirement for PCNA in DSB repair (15) are shown in **Table 1**, along with the results of those crosses. The *sn* gene is located on the X chromosome at cytological position 7D1,2. *mus209B1* is a temperature-sensitive lethal PCNA mutant that exhibits sensitivity to ionizing radiation and methyl methanesulfonate at temperatures permissive for growth (18). It is located on chromosome 2. $\Delta 2-3(68C)$ is inserted on the left arm of chromosome 3.

The results of cross 1 show that the combination of *snw* and transposase ($\Delta 2-3$) is lethal to *mus209B1* homozygous males and females (genotypes **a** and **c**), demonstrating that PCNA is essential for DSB repair. In cross 2, which is the reciprocal cross, *mus209B1* homozygous females (genotype **g**) were killed, but homozygous males (genotype **e**) survived since they lacked the transposase target, *snw*. Note, too, the dominant nature of the lethality in the case of the female homozygous offspring: *snw/+; mus209B1/mus209B1; Δ2-3/+* females were killed despite having had a wild-type X chromosome in addition to the *snw*-bearing homolog. For any test of this sort, it is advisable to set up reciprocal crosses and to include, as a control, crosses without $\Delta 2-3$ (not shown).

3.2. Cytological Analysis of DSBs

Clues regarding the in vivo function of a repair protein can often be obtained from cytological analyses of mutants. For example, a high frequency of broken

mitotic chromosomes would be indicative of a defective DNA damage checkpoint. A simple and effective way of analyzing mitotic chromosomes of *Drosophila* is through squashed preparations of brains from 3rd instar larvae (23).

1. Select a larva of the desired genotype (see **Note 3**), and wash in 0.7% NaCl. Transfer it to a fresh drop of saline.
2. To dissect out the brain, grasp the mouthparts and the middle of the larva with two pairs of forceps and pull. The brain usually comes out with imaginal disks and other tissues attached. Carefully dissect these from the brain. (See **Note 4**.)
3. Transfer the brain to a microscope slide, and add a drop (~10 μ L) of 45% acetic acid. Leave for 30 s. (See **Note 5**.)
4. After 30 s, carefully remove the liquid by absorbing it into a tissue.
5. Add a drop of 3% orcein/45% acetic acid, and leave for 3 min.
6. After 30 s, carefully remove the liquid with a tissue.
7. Add a drop of 60% acetic acid, and then immediately remove the liquid with a tissue.
8. Add a drop of 3% orcein/60% acetic acid, and then immediately cover it with a cover slip (see **Note 5**).
9. Carefully insert the slide into a folded piece of 3MM paper, and squash it either by pressing hard with your thumbs on opposite corners of the cover slip, or by standing on it with the ball of your foot or your heel. If using the foot method, place the slide (sandwiched in 3MM) on a hard clean floor, cover it carefully with a piece of wood, and stand on that. Press hard for 1 min, and then gently release the pressure.
10. Seal the edges of the cover slip with nail polish. Analyze the mitotic figures under a phase-contrast microscope.

An example of a mitotic figure from an *snw/+; mus209B1/mus209B1; Δ 2-3/+* larval brain prepared in this way (using the foot method) is shown in **Fig. 2**.

4. Notes

1. A number of different, Δ 2-3 strains exist, each with the Δ 2-3 element stably integrated at a different genomic location. The most widely used line is designated Δ 2-3(99B), the suffix “99B” indicating its cytological location near the tip of the right arm of chromosome 3. Δ 2-3 elements located on balancer chromosomes are now available (consult FlyBase) and may prove advantageous in some cases. I prefer to use Δ 2-3(68C), since it causes a higher level of killing than Δ 2-3(99B) in this assay, at least in the case of *mus209* mutants.
2. Depending on the origin of this strain, the *snw*-bearing chromosome may carry a third P element at 7D4-5 not associated with *snw* at 7D1-2 (22).
3. Being able to identify the desired genotype in larvae may require the construction of appropriately marked strains. For example, *snw/+; mus209B1/mus209B1; Δ 2-3(68C)/+* larvae for cytological analysis (**Fig. 2**) were generated by crossing *+/+; mus209B1; Δ 2-3(68C)/TSTL14* females to *snw/Y; mus209B1, Bc/CyO; +/-* males. *Bc* (*Black cells*) is a dominant larval marker. The balancer chromosome *TSTL14* was derived from a reciprocal translocation involving chromosomes 2 and 3 (see **ref. 24**) and forces the cosegregation of *mus209B1* and Δ 2-3(68C) in

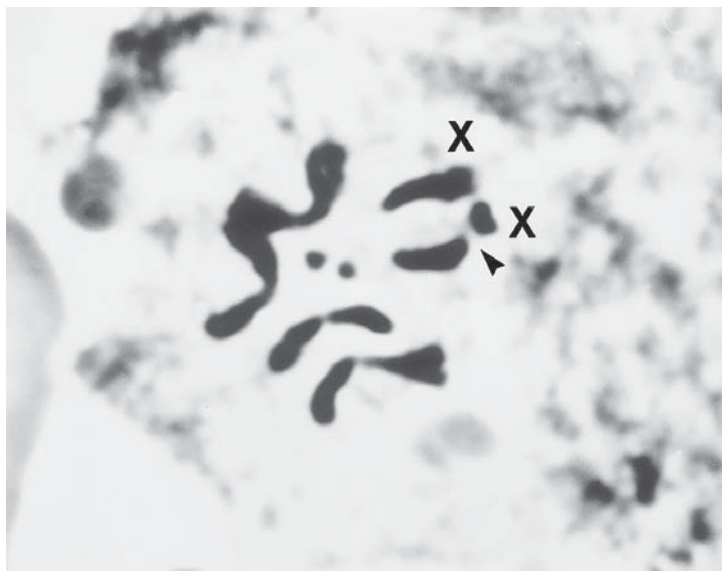


Fig. 2. Orcein-stained mitotic chromosomes in a neuroblast from an *snw/+; mus209B1/mus209B1; Δ2-3/+* larva analyzed by phase-contrast microscopy. The X chromosomes are labeled. The two dot-like chromosomes at the center of this mitotic figure are chromosomes 4. The two pairs of large metacentric chromosomes are chromosomes 2 and 3. Note the isochromatid break (arrowhead) at the distal end of one of the X chromosomes. This break is consistent with the position of the *sn* locus. Adapted from ref. (15) and used with permission of Oxford University Press.

that cross. *TSTL14* also carries the dominant larval marker *Tb* (*Tubby*), which therefore allowed *snw/+; mus209B1/mus209B1; Δ2-3(68C)/+* larvae to be identified by their Bc, non-Tb phenotype and gonadal anatomy. This elaborate scheme was necessary because *mus209B1* females are sterile. Simpler designs are possible for fertile mutants.

4. Colchicine treatment and hypotonic shock can make visualization of chromosomes easier. After dissecting out the brain, transfer it to 0.7 % NaCl containing 0.5 μg/mL colchicine, and incubate in the dark for 45 min to 2 h. Wash in 0.5% trisodium citrate, and proceed to **step 3**.
5. For best preparations, clean the slides and cover slips with ethanol just before use, and keep them dust-free by using Dust-Off® (Falcon Safety Products, Inc., Sommerville, NJ).

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Analyzing Double-Strand Repair Events in *Drosophila*

Gregory B. Gloor, Tammy Dray, and Kathy Keeler

1. Introduction

Targeted manipulation of the genome is used to analyze gene expression, genome structure and protein structure. In yeast it has long been possible to introduce sequences into predetermined sites in the genome by double-strand break (DSB) repair (**1**). Until recently, such specificity has eluded investigators that use *Drosophila* as their model organism. Such a gene-targeting system is now available in *Drosophila*. The system is based on the repair of a specific DSB made by P element excision (**2–12**).

The repair of the double-strand DNA break that results from P element excision occurs when the broken DNA ends find, invade, and prime DNA synthesis from a homologous donor sequence located elsewhere in the nucleus (**13,14**). In the process outlined in **Fig. 1**, information from the donor DNA molecule is transferred into the break site without the donor molecule being affected. Therefore, gene conversion is the predominant result of gap repair in *Drosophila*. Repair is extremely efficient—in most situations repair of about 1% of the available DSBs proceeds by gene conversion from the donor, but the repair efficiency varies with donor position and sequence composition (range 0.1–50%) (**4,5,7,11,14,15**). The donors in these experiments carry a *white* gene with multiple single-base alterations that alter the *white* gene restriction map. This enables the detection of conversion events by PCR amplification and restriction mapping of the amplimers.

This chapter will describe how to collect and analyze two different products that result from the repair of P element excision events in *Drosophila* (**Fig. 1**). The first product has DNA sequence flanking the excised P element replaced by donor sequence (**6,10,14**); we will refer to this as an external conversion

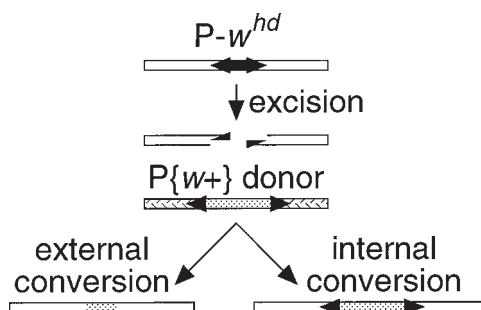


Fig. 1. P element-induced gap repair in *Drosophila*. The P-*whd* element is a non-autonomous P element inserted in exon 6 of the *white* gene. The donor element is a modified *white* gene carried in the genome as a P element transgene and is located at an ectopic site. Following excision, the broken chromosome ends may retain some of the sequence of the P-*whd*-inverted terminal repeats. The repair product that results is dependent on whether the P sequences are retained or lost at the break site. If the P sequences are lost, then the flanking *white* gene sequence is used to conduct a homology search. In the example here, the ends find the ectopic *white* gene and serve as primers to copy sequence from it. The result is a gene conversion in which the *white* gene sequence external to the original P-*whd* is derived from the ectopic donor. If the P sequences are not lost, then they are used to conduct a homology search. In this instance, the sequences internal to the P-*whd* element are replaced with those derived from the donor element. Gene conversion by both methods is about equally frequent at the *white* locus.

event. In the second product, a different P element replaces the P-*whd* element precisely (6); we will use the term internal conversion to refer to these events. We will describe our genetic and molecular screens for gap repair at the *white* locus.

When the goal of the experiment is external conversion, there are several important parameters to consider. First, the donor sequence must have sufficient homology for recognition by the broken ends. The amount of homology required depends on the sequence to be targeted. Single-base alterations need as little as 115 bp of flanking homology for efficient targeting (11), whereas 8-kbp insertions need about 500 bp of flanking homology (15). These figures are estimates of the minimum amount of flanking homology required for efficient targeting. Second, it is important that the donor sequence be isogenic with the break site, except for the particular sequence that the investigator wishes to introduce into the genome. Each difference between the donor sequence and the sequence at the break site will reduce the efficiency of conversion substantially (11,15). These two points are very important to remember when designing a donor sequence, and prudence suggests maximizing the nature and extent of homology whenever possible. Third, we have found that conversion from an

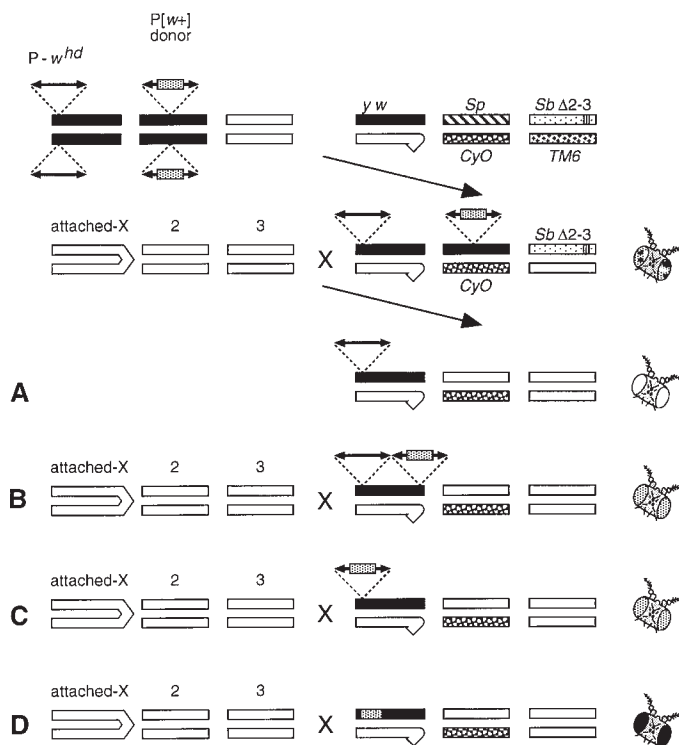


Fig. 2. Genetic screen for gap repair in *Drosophila*. In the parental cross (top), females homozygous for the *P-w^{hd}* element and the *P{w⁺}* donor element are mated to male flies that carry the *CyO* second chromosome and the *Sb Δ2-3* third chromosome. Their male progeny contain the three components required for gap repair: the *P-w^{hd}* chromosome, the donor element heterozygous with the *CyO* balancer chromosome, and the *Δ2-3* transposase source. In the F₁ cross (second from top), these males are mated individually to three to five attached-X females, and their male progeny are collected. These F₂ progeny contain X chromosomes on which gap repair has occurred, and there are four general classes, A–D, which are explained in the text. Only those males that carry the *CyO* chromosome and lack the *Δ2-3* chromosome are kept. The F₂ males in classes B–D are mated individually to attached X females to assess the linkage of the eye color phenotype.

injected plasmid template occurs as efficiently as conversion from a template inserted at an ectopic site in the genome (5). This may be useful for those labs that perform microinjection of *Drosophila* embryos routinely.

The analysis of a putative gap repair event proceeds through several steps. The first three steps use a genetic screen and the last two steps use molecular analysis. These steps are outlined in **Figs. 2** and **3**. We have designed our

Molecular Screen

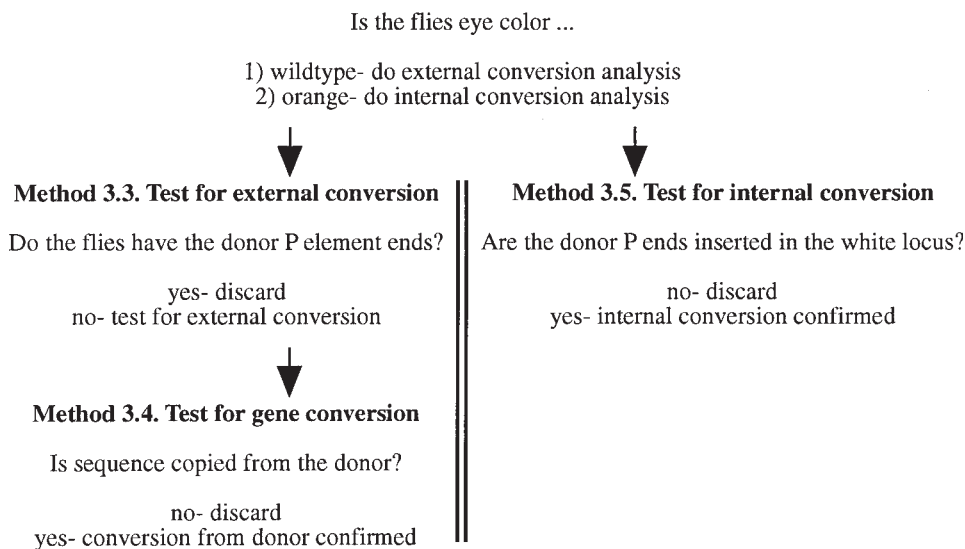


Fig. 3. Molecular screen for gap repair in *Drosophila*. The *white* gene is analyzed by a PCR-based molecular screen for those *Drosophila* lines in which the change in eye color phenotype maps to the *X* chromosome. There are two separate analyses required for external and internal conversion events. External conversions do not show linkage between the donor P element end and the *white* locus. Therefore, we conduct a PCR screen for the donor left and right ends. Those that lack the donor ends are kept, and the region flanking the P-*whd* excision site is amplified by PCR. The amplicon is digested with an appropriate restriction endonuclease and analyzed by agarose-gel electrophoresis to detect copied donor sequences. Internal conversions contain the donor P element ends inserted in place of the P-*whd* ends in the *white* locus. A PCR amplification to confirm linkage of both ends is diagnostic of an internal conversion event.

procedure to optimize and simplify the analysis at each step. We design the initial fly matings so that the chromosome with the donor sequence segregates away from the chromosome on which gap repair is occurring (Fig. 2). Targeting events are relatively simple to identify if the repair event confers an easily detected phenotype. In our case, external conversions confer a wild-type eye color to the flies, and internal conversions confer an orange eye color (6,13,14). Third, a mating between the flies containing the putative events and tester

strains determines if the phenotype segregates with the appropriate chromosome. Fourth, the putative events are tested for the presence or absence of the donor by PCR. Finally, an analysis of the potential events by PCR amplification is followed, if necessary, by restriction endonuclease digestion.

2. Materials

Water for the solutions should be of the best-quality available. All chemicals: Life Technologies Industries Ultrapure (Gaithersburg, MD), Sigma (St. Louis, MO): Molecular Biology grade or equivalent unless noted otherwise.

2.1. Genetic Analysis

1. Dissecting microscope.
2. CO₂ anesthetizing apparatus.
3. Fly food is the standard cornmeal, sucrose, and agar food.
4. Fly strains (see **Note 1**):
 - a. *y whd f*; *P{w+ donor}* ; +
 - b. *y w*; *CyO/Sp*; *Sb Δ2-3(99B)/TM6*
 - c. *C(1)DX, y w f*; +; +

2.2. DNA Preparation

1. 500-μL polypropylene tubes (Eppendorf, Hamburg, Germany).
2. Proteinase K (Promega, Madison, WI): 20 mg/mL in water. Keep at -20°C.
3. Squishing buffer: 10 mM Tris-HCl, pH 7.8, 25 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100. This may be kept at room temperature for several months. To minimize contamination it is important that the squishing buffer be made up with solutions and glassware that have never been in contact with plasmids or PCR products. Proteinase K is diluted 1:10 in squishing buffer just before use.
4. Refrigerator for 4°C incubation.
5. Perkin-Elmer DNA Thermocycler or dry bath incubators at 37°C and 95°C. It is important that the entire wall of the tube be in complete contact with the incubator.

2.3. PCR

1. *Taq* polymerase (Perkin-Elmer, Norwalk, CT; Amersham Pharmacia Biotech, Uppsala, Sweden) at 5 U/μL.
2. 10X PCR buffer is composed of 200 mM Tris-HCl, pH 8.6, 500 mM KCl, 0.1% Triton X-100.
3. 50 mM MgCl₂.
4. The four deoxyribonucleotide triphosphates are purchased as ultrapure solutions from Pharmacia at a concentration of 100 mM. Equal amounts of each dNTP are mixed and frozen as 50-μL aliquots at -70°C. Any dNTP mix not used in 9 mo is discarded. Each dNTP solution is diluted by adding 950 μL of distilled water before use. The final concentration is 1.25 mM of each dNTP in the mix. This diluted dNTP mix can be stored at -20°C for 4–8 weeks before use.

5. Oligonucleotides are purchased as deblocked crude pellets and are used without further purification. They are resuspended at a final concentration of 200 μM in water and kept at -70°C , where they are stable for at least 1 yr. Working stocks of oligonucleotide primers are diluted to a concentration of 20 μM and kept at -20°C until use. They are stable for several months under these conditions. The seven oligonucleotides required for these experiments are:

a. GGTGTCGTACCTCTCATGG	ef+
b. ACAGCGAAAGAGCAACTACG	Hi-
c. AGCCAAGCTTTGCGTACTCG	wPL1
d. GCAGCCTTCCACTGCGAATC	P310
e. GGTGGCGCGATCTCGCGCTCT	I+
f. AAGAGATAGCGGACGCAGCG	CasRt2
g. GAGTGTCGTATTGAGTCTGAG	20108
6. Light mineral oil.

2.4. Restriction Endonuclease Digestion

1. *Hae*III restriction enzyme (New England Biolabs, Beverly MA; Pharmacia or Promega).
2. 0.1 M MgCl_2 , made up in distilled water and autoclaved.
3. 0.25 M Tris-HCl, pH 7.8, made up in distilled water and autoclaved.
4. 5 M NaCl, made up in distilled water and autoclaved.
5. 10X agarose gel-loading buffer: 10% Ficoll 70, 1% sodium dodecyl sulfate, 20 mM EDTA, 0.1% bromophenol blue.

3. Methods

3.1. Genetic Analysis

Figure 2 shows the genetic screen for potential DSB repair events (*see* **Notes 1** and **2**). In the parental cross, female flies that carry the *P-whd* element on the *X* chromosome and the *P{w+}* donor element on the second chromosome are mated to males that carry the *CyO* second chromosome balancer and the *Sb* $\Delta 2-3$ transposase source. The male F_1 progeny of this mating contain the *P-whd* element, the *P{w+}* donor element and the transposase source. They generally have a mosaic eye phenotype caused by somatic transposition of the *P-whd* and the donor elements. These males are mated individually to three to five attached-X female flies. The purpose of this cross is to recover the putative F_1 germline events on the *X* chromosome of the F_2 progeny and at the same time to segregate away the original donor element and the transposase source.

The F_2 males inherit their *X* chromosome from their father because of the compound *X* chromosome, and we keep only those males that lack both the original donor element and the transposase source (**Fig. 2**). The progeny have three main phenotypes that are associated with the four possible events.

The first phenotypic class (**A**) is white-eyed flies, which represents no change at the *white* locus. Flies in this class make up the vast majority of the progeny and are not analyzed further. The second class of progeny has an orange eye color, and represents either transposition of the donor to another site in the genome (**B**), shown in **Fig. 2** is a transposition to a random site on the X chromosome), or replacement of the P-*whd* element with the donor element (**C**). These two events have similar phenotypes. The final phenotype has a wild-type eye color (**D**). Flies in this phenotypic class are either precise excisions of the P-*whd* element, or transpositions of the donor element to a site in the genome that permits a high level of expression of the *white* gene in the donor element. Again, these possibilities must be sorted out by further analysis.

The F₂ males that have an orange or wild-type eye color are mated to attached-X virgin females, and the segregation of the eye color phenotype is followed. If the eye color change segregates with the X chromosome, the flies are maintained as a line, and DNA is prepared for molecular analysis to determine if an internal or external gene conversion occurred (**Fig. 3**). The flies are discarded if the eye color phenotype segregates with an autosome or the Y chromosome.

3.2. DNA Preparation

The objective here is to prepare DNA from candidate flies for analysis by PCR amplification. This protocol is modified slightly from one published previously (**16**). We immobilize the flies by chilling them and thoroughly mash them with squishing buffer. Flies may be collected beforehand and frozen for several weeks before squishing. We have found that the addition of nonionic detergent increases the reproducibility of the procedure without any apparent effect on the long-term storage of the DNA. It is important to minimize the time that the DNA is at 95°C, because it deteriorates rapidly at this temperature. In some cases, such as inverse PCR, it is desirable not to denature the DNA. When this is the case, the proteinase K is inactivated by adding 1 µL of phenylmethyl sulfonyl fluoride (PMSF) followed by heating to 65°C. The DNA prepared by either method is stable at 4°C for at least 1 mo without deterioration. Previously frozen DNA samples do not support PCR amplification. Addition of more than 1 µL results in poorer amplification owing to inhibitors in the preparation.

1. Place single, CO₂-anesthetized male flies in 500-µL polypropylene tubes. Place the tubes on ice or at -20°C until the flies stop moving.
2. Take up approx 50 µL of squishing buffer plus proteinase K in a yellow pipet tip, and place 5–10 µL of the buffer into the bottom of the tube.
3. Macerate the flies by vigorous strokes with the end of the pipet tip, and expel the remainder of the liquid. Alternatively, the entire 50 µL can be placed in the tube,

and the fly macerated with a plastic or metal pestle that fits snugly into the bottom of the tube. Wooden toothpicks are not suitable. Pestles that are used instead of disposable yellow pipet tips can be reused by washing them in 0.1 *N* HCl overnight and rinsing them with water.

4. Incubate the tube at 37°C for at least 20 min, followed by an incubation at 95°C for 3 min.
5. Briefly centrifuge the DNA preparation to bring any condensate down from the sides of the tube. DNA preparations may be stored for several months at 4°C. If the water evaporates, the DNA can be rehydrated by the addition of 50 µL of water to the sample. Do not freeze. Each DNA sample prepared in this way contains about 750 haploid genomes/µL (range 57–1800, *n* = 4) (16).

3.3. PCR Screen for Donor P Element Ends

This PCR screen tests for the presence or absence of donor P element ends in the flies with a wild-type eye phenotype (Fig. 3). External conversion products have undergone gene conversion external to the *P-whd* element. Thus, they will have lost the *P-whd* element following gene conversion. In addition, these events will not have the donor P element ends. The presence or absence of donor P element ends are tested for with two separate PCRs, one for the left donor end using primer pairs 20108/CaRt2 and one for the right donor end using I+/3645. It is important to analyze at least four flies from each line. This ensures the detection of most cryptic donor elements inserted on the autosomes.

1. Perform PCRs in a volume of 15 µL in a 500-µL Eppendorf tube. The common components of the reaction mix, except the primers, are made up as a master mix. The master mix contains the following components added in the following order:
 - a. Water to 12.5 µL final volume
 - b. 5 mM dNTP mix 2.4 µL
 - c. 10X PCR Buffer 1.5 µL
 - d. 50 mM MgCl₂ 0.45 µL
 - e. *Taq* Polymerase 0.12 µL at 5 U/µL
2. Aliquot 12.5 µL of mix into a 500-µL Eppendorf tube.
3. Add 1 µL of DNA as prepared in **Subheading 3.2**.
4. Overlay with one drop of light mineral oil dropped into the tube from a 200-µL volume pipet tip.
5. All PCRs use a hot-start protocol (17). To do this, we place the tubes in a thermocycler and incubate at 85°C for at least 2 min.
6. Mix the primers 20108 and CaRt2 or I+ and 3645 together in equal proportions. Add 1.5 µL of the primer mixture to the tubes by placing the tip at the bottom of the tube while it is in the thermocycler and expelling completely.
7. The first PCR cycle has the following parameters:
 - Denature at 95°C for 1 min.
 - Anneal at 72°C for 1 min.
 - Extend at 72°C for 1.5 min.

8. All PCRs use a touchdown protocol (**18**). To do this, drop the annealing temperature 1°C for each subsequent cycle until an annealing temperature of 55°C is reached.
9. Amplify for a further 25 cycles with the conditions:
 - Denature at 95°C for 1 min.
 - Anneal at 55°C for 1 min.
 - Extend at 72°C for 1.5 min.
10. Analyze by running the reaction products on a 1.4% agarose gel. In this case, we are looking for lack of an amplicon, so proper positive controls are crucial. We always include at least two independent fly preparations that contain donor sequence as positive controls.

3.4. PCR Screen for External Conversion Events

In the absence of a donor element, the only *white* sequence available for amplification is the *white* locus. This PCR uses the primers ef+ and Hi- to find precise excisions of the P-*whd* element (**Fig. 4**). If these excisions occurred by gene conversion from the donor element, the *white* locus will have one of the characteristic base alterations incorporated at this site. These conversion events are recognized by a characteristic restriction endonuclease digestion pattern of the amplicons. Our donor *white* genes lack the *Hae*III site located at the position corresponding to the DSB at the *white* locus (**Fig. 4**). As a result almost all (>98%) of the gene conversions copy this base alteration, making it a useful marker for gene conversion. Heterologous sequences can be cloned into the multiple cloning site (MCS) and converted into the *white* locus (see **Note 3**).

1. Prepare the PCR tubes as described in **Subheading 3.3., steps 1–5**.
2. Mix primers ef+ and Hi-, and add to the reaction mix as in **Subheading 3.3., step 6**.
3. Use the same hot-start and touchdown PCR protocol as described in **Subheading 3.3., steps 7–9**. Carry out all subsequent manipulations in a different room to prevent contamination of future reactions.
4. Make up a digestion mix for the enzyme to be tested. For *Hae*III, in each 5 µL of digestion mix, add the following:
 - a. 1.5 µL of 100 mM MgCl₂.
 - b. 1.5 µL of 250 mM Tris-HCl, pH 7.8.
 - c. 1 U of *Hae*III.
 - d. Distilled water to 5 µL.
5. Add 5 µL of digestion mix under the oil. Mix by pipeting up and down several times or by gently flicking the tube with a finger until the contents are mixed. The final concentration of the salts is 9 mM MgCl₂ and 37 mM KCl, and the final pH is 8.3. Similar mixes can be made that provide an acceptable environment for almost any restriction endonuclease.
6. Incubate at 37°C for at least 1 h.
7. Set the pipeter to accept a volume of 20 µL, and take up 2–3 µL of 10X gel-loading buffer. Suck the completed restriction digest into the tip, and mix by

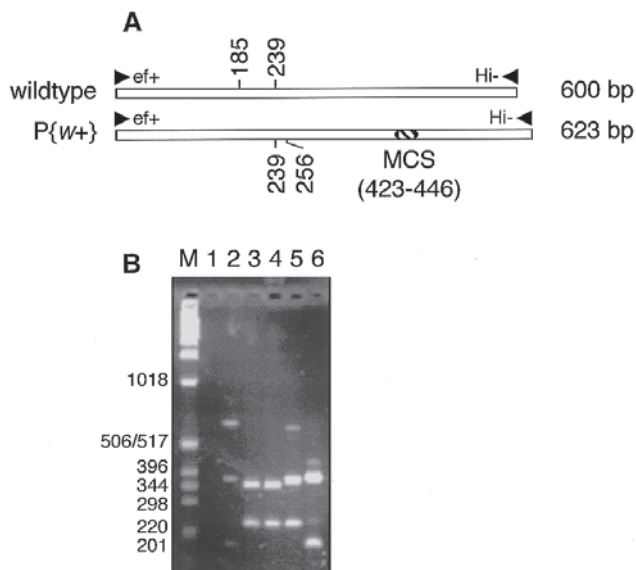


Fig. 4. Confirmation of external conversion events. All our P{w+} donor elements contain single-base alterations that allow identification of donor sequences copied into the *white* locus. (A) shows the *Hae*III restriction map of a 600 bp fragment of the canonical *white* gene sequence and the *white* gene sequence as it is found in the P{w+} donor element. In this example, the donor element contains one *Hae*III site (position 256) that is lacking in the normal *white* gene, and has another *Hae*III site (position 185) removed. The 8-bp target site of the P-*whd* element overlaps with the *Hae*III site at position 185. MCS in P{w+} is the MCS. In (B) the 600-bp region that flanks the P-*whd* excision site in the genomic *white* gene was amplified by PCR with ef+/Hi- and digested with *Hae*III and run on a 3% agarose gel. Lane 1 is an amplification that did not contain *white* gene sequence, and lane 2 is an amplification from a fly that contained P-*whd*. Lanes 3–6 are amplifications from putative gap repair events digested with *Hae*III. Lanes 3 and 4 are converted for the donor sites at positions 185 and 256, lane 5 is converted for only the site at 185, and lane 6 is unconverted.

expelling under the oil two to three times. Load onto a 3% agarose gel. In this case, we are looking for the restriction digest pattern of the amplicon, so proper positive controls are crucial to ensure the PCR worked as desired. We always include at least one positive and one negative control for gene conversion of the site we are interested in. **Figure 4** shows the results of a successful screen for conversion of two sites next to the P-*whd* insertion site.

3.5. PCR Screen for Internal Conversion Events

This screen tests for linkage between the *white* locus and the donor P element ends (**Fig. 3**) (6). Two separate PCRs are performed. The first screen tests

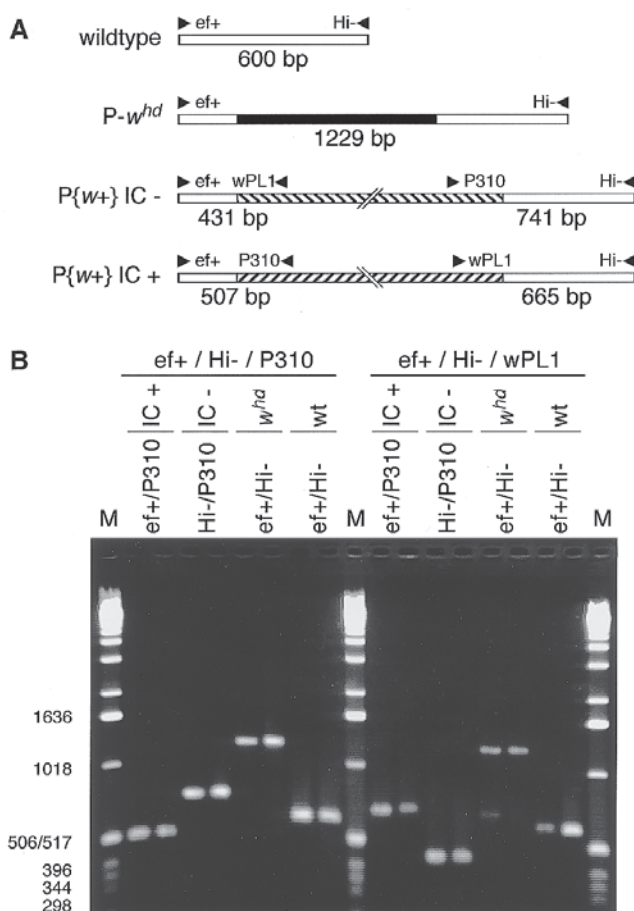


Fig. 5. Internal conversion screen. Flies with orange eyes showing *X* chromosome linkage with the eye color phenotype were subjected to an internal conversion screen. This screen uses three oligonucleotide primers concurrently to test for replacement of the P-*whd* element with the P{*w+*} donor transposon. The initial screen uses the primers ef+, Hi-, and P310. The oligonucleotide binding sites and sizes of the amplimers are shown in (A), and a sample gel showing the sizes of the amplimers is shown in (B). DNA samples in which the 741- or 507-bp amplimers are observed are tested for amplification with ef+, Hi-, and wPL1. In a successful internal conversion, the size of the second amplimer should correspond to the size expected in (A). For example, if the size of the amplimer in the first screen was 741 bp, then the size of the second amplimer should be 431 bp. This would correspond to a P{*w+*} IC- event.

for the insertion of the right donor end in the *white* locus with primers ef+, Hi-, and P310. This screen can detect such insertions in either orientation giving a product of either 507 bp (ef+ – P310) or 741 bp (Hi—P310) (**Fig. 5**). The second

PCR tests for the left donor end inserted in the *white* locus with primers ef+, Hi−, and wPL1. This secondary test can also detect insertions in either orientation, giving products of either 431 bp (ef+–wPL1) or 665 bp (Hi−wPL1) (**Fig. 5**). Only those events in which each end is linked uniquely to the flanking primers are kept.

1. Prepare the PCR tubes as described in **Subheading 3.3., steps 1–5**.
2. Mix primers ef+, Hi−, and P310 in equal proportions and add 2.3 μ L to the reaction mix.
3. Amplification uses the same hot-start and touchdown PCR protocols as in **Subheading 3.3., steps 7–9**.
4. Run the completed PCRs on a 1.4% agarose gel.
5. When a putative event is identified, another PCR is performed with the primers ef+, Hi−, and wPL1 to determine if the other end is inserted in the *white* locus. **Figure 5** shows the results of a successful screen for replacement of the P-*whd* element with the donor element.

4. Notes

1. It is most convenient to assemble the three required components if the break site, the donor, and the transposase source are each on their own chromosomes. The fly strains used here are optimal for conversion to the X chromosome using a third-chromosome-linked transposase source. If the desired product has a clearly distinguishable phenotype from the starting components, then it is possible to combine the break site and donor in cis to achieve a significantly greater conversion frequency (2).
2. Since the DSB repair events require a P element excision, an important parameter is the mobility of the original P element. The P-*whd* element transposes about once per fly generation per gamete sampled (13). Thus, it is assumed that there is one DSB generated per transposition. It is important that the investigator determine the transposition rate of the element making the break. For example, if a transposon jump is found in 10% of the gametes, then the transposition rate is about 10% of the P-*whd* rate, and 10 times more progeny will have to be screened than if the same experiment was attempted at the *white* locus.
3. We have inserted the *yellow* and *forked* genes in the MCS and targeted these genes to the *white* locus. When the goal is the targeting of such a heterologous sequence to the *white* locus, the PCR screen may need modification because the insert may be too large to permit amplification from ef+ to Hi− under the conditions described in the text. There are two possible modifications. First, the PCR screen can be modified so that an oligonucleotide specific for the heterologous insertion produces a specific product following PCR amplification between ef+ or Hi− and the specific oligonucleotide. This will show linkage between the *white* locus and the heterologous sequence. Second, a long PCR can be performed to amplify across the heterologous insertion. Our long PCR protocol is derived from Barnes and is described below (19,20). The following additional materials are required:

- a. DNA polymerase mix: 250 U *Taq* polymerase, 2 U *Pfu* polymerase (Stratagene).
- b. 10X Low-salt PCR buffer (for long PCR <9 kb): 200 mM Tris-HCl, pH 8.75, 100 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, 1% Triton X-100, 1 mg/mL BSA.
- c. 10X High-salt buffer (for long PCR >9 kb): 200 mM Tris-HCl, pH 9.2, 600 mM KCl, 20 mM MgCl₂.
- d. Oligonucleotide primers for long PCR that flank the P-*whd* and multiple cloning site:
 - i. 2362, GCACATCGTCGAACACCACG
 - ii. H-, GTGTTTTATGTACCGATAAACGAG

To each tube add: 8.87 μ L of H₂O, 2.5 μ L of dNTPs, 1.6 μ L of 10X reaction buffer, 0.16 μ L of DNA polymerase mix. The fly DNA, oil, and oligonucleotide primers are added as before. As a general rule of thumb, about 1 min of extension time/kb is sufficient. The reactions are hot-started as usual. We have found that the following conditions give good amplification. Program A is 30 PCR cycles with denaturation at 99°C for 30 s, annealing at 66°C for 2 min, and extension at 68°C for 15 min. This protocol works well for amplifications that include up to 8 kb of DNA. Program B is 30 PCR cycles with denaturation at 99°C for 30 s, annealing at 62°C for 2 min, and extension at 68°C for 20 min. This protocol works well for amplifications that include up to 13 kb of DNA.

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Expression of I-Sce I in *Drosophila* to Induce DNA Double-Strand Breaks

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1. Introduction

Generation of double-strand breaks (DSBs) in chromosomal DNA induces repair machinery of a cell, and is also a necessary step for recombination events. A system for the directed introduction of DSBs into a genome could substantially facilitate progress in understanding DSB repair mechanisms and could be used for efficient gene targeting. The most successful attempts toward this goal in *Drosophila* have utilized the P element transposition system. However, directed introduction of DSBs is still neither highly precise nor efficient, probably in part owing to the innate properties of the P element transposase, which although being a site-specific DNA binding protein, also has an affinity for nonspecific DNA sequences in vitro (**1**). As a result, DSBs generated by P element transposase are distributed randomly in the *Drosophila* genome with the highest frequency close to or at the P element ends. Site-specific endonucleases with sufficiently long recognition sequences potentially could provide a solution to this problem. Among the most specific is the I-Sce I endonuclease. It recognizes an 18-bp nonpalindromic sequence (*see* Chapter 37) and has very low tolerance to nucleotide substitution. Theoretically, this recognition site should appear only once in every 6.87×10^{10} bp, which exceeds the size of the *Drosophila* genome by about 400 times.

I-Sce I was the first discovered member of a vast family of homing endonucleases encoded by mobile introns. There are several excellent reviews discussing properties of these enzymes (**2–4**). I-Sce I is not a recombinase, so its potential for chromosome engineering is different from that of systems

with target-site requirements on both host and donor molecules (e.g., FLP/FRT or Cre/lox systems). The result of the enzymatic activity of recombinases is predetermined. FLP, for example, excises any DNA sequence between direct FRT sites and inverts it if the FRT sites are inverted with respect to each other. Another important difference is in the mechanism of interaction with the substrate molecules. Recombinases appear to be required in stoichiometric rather than catalytic amounts (5) and are covalently bound to the DNA intermediates (6). The reaction rates exhibit a strong dependence on recombinase concentration, with low turnover numbers, even when the protein is present in excess relative to available recombination sites. All this may suggest that strong association of recombinases with DNA template, and their close position to the cleavage site, can shield DNA breaks from the recombination/repair machinery of the genome. Low turnover was observed for I-Sce I endonuclease as well, though it is not clear whether it could be attributed to the fast enzyme decay or the slow product release (7). More important is that I-Sce I apparently does not bind covalently to the DNA substrate, though the enzyme shows an asymmetric binding affinity for the recognition site. It binds more strongly and releases more slowly the downstream half of the recognition site. This asymmetry may be important for the repair of DSBs and design of gene-targeting systems, so that relative orientation of the I-Sce I cleavage site and the reporter construct may produce different results. In addition to this differential binding, I-Sce I probably is not involved in any recombination events following the DSB cleavage.

2. Materials

Two constructs are necessary for a functional assay of the I-Sce I activity: one that can provide a stable source of I-Sce I, and another, the reporter construct, which can provide a means to monitor the DSB introduced into the DNA by I-Sce I. This reporter construct should comply with several requirements:

1. It necessarily contains the I-Sce I recognition sequence.
2. It should be easily “scorable” at the phenotypic level, i.e., the phenotype of progeny bearing the altered reporter construct should be reasonably distinct from that of progeny carrying the unchanged reporter construct.
3. The design of the reporter construct should allow for relatively simple and straightforward subsequent molecular analysis of the individual chromosomes (i.e., conveniently located primer sites for PCR, unique DNA sequences for Southern blot analysis, and so forth). In addition, the reporter construct may include any other sequences, depending on the requirements of the particular experiment, such as stretches of homologous DNA in inverted or direct orientation, on one or both sides of the I-Sce I recognition sequence.

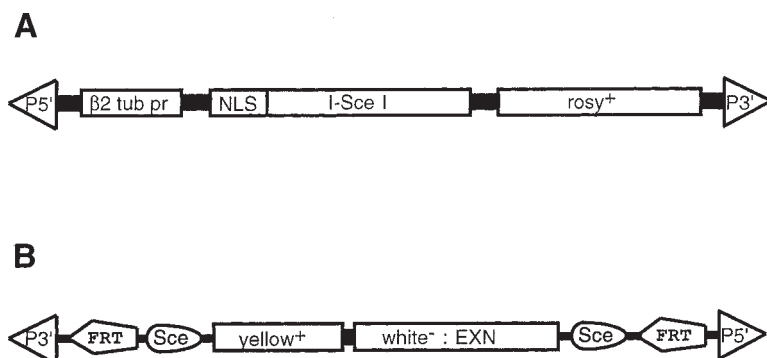


Fig. 1. Schematic maps of the constructs used for the functional assay of I-Sce I activity in the *Drosophila* genome. (A) Expression construct. (B) Reporter construct. See text for details.

3. Method

3.1. Design of a Vector for Expression of I-Sce I in *Drosophila*

I-Sce I expression construct was introduced into the *Drosophila* genome by P element transformation with *ry+* as a selectable marker (Fig. 1A) (8). I-Sce I endonuclease is a product of the class I intron of the mitochondrial 21S rRNA gene of *Saccharomyces cerevisiae*. Its genetic code differs from the universal code. In order to ensure an efficient synthesis in the *Drosophila* genome of a polypeptide identical in size and sequence to the I-Sce I from yeast mitochondria, we made use of the I-Sce I DNA modified by the oligonucleotide-directed mutagenesis (9). In that DNA, all nonuniversal as well as rare codons were substituted by universal codons (that account for approx 30% of all the codons) (10; see Note 1).

I-Sce I is expressed in our system in the male germline cells under the β 2-tubulin promoter. The reason for choosing this particular promoter is that it provides several unique advantages. It drives expression of the β 2-tubulin protein only in cells of the male germline. Expression first occurs at the late primary spermatocyte stage (11), after gonial cells have completed mitotic divisions, and mature primary spermatocytes are ready for the meiotic divisions. The spermatocyte-specific expression is controlled by a 14-bp *cis*-acting element (12; see Note 2).

After the P element directed transformation of the I-Sce I expression construct into the *Drosophila* genome, several lines with the construct located on the third chromosome were selected. Lines II.9 and L9.8 are homozygous viable, and II.5 and L9.2 are homozygous lethal, and were balanced over the *TM2,Ubx* balancer chromosome. All lines are available from the authors upon

request (8). Expression of the functional I-Sce I enzyme has no apparent influence on viability or fertility. Stocks can be easily maintained at 25 and 18°C.

3.2. Design of a Reporter Construct

A schematic map of the reporter construct used for the functional assay of the I-Sce I enzyme is shown in **Fig. 1B (8)**. The construct was made on the basis of the Carnegie4 transformation vector. 3' and 5' P element sequences from this vector flank the entire construct. Two FRT, or recombination target sequences for the yeast site-specific recombinase FLP, were introduced on both sides of the construct in direct orientation with respect to each other, and were placed between P element sequences and recognition sites for I-Sce I endonuclease. These FRT sites provide stretches of homologous DNA sequences. Selectable markers were inserted between two directly oriented I-Sce I restriction sites. One is a genetically engineered cuticle pigmentation gene, *yellow* (13). It contains the entire coding sequence as well as enhancer sequences directing expression of the gene in adult body cuticle, wing blade, larval mouth parts, and denticle belts. The second marker is a copy of the *white* gene with artificially introduced additional restriction sites for several restriction enzymes (8).

The reporter construct was introduced into the *Drosophila* genome by the P element-directed transformation. The recipient line has a deletion of the endogenous *yellow* gene and adjacent sequences, making the *yellow* gene from the reporter construct a unique sequence (13). Several independent transformant lines were selected with the reporter construct inserted on the X chromosome and the second chromosome. Line H3.3 is homozygous viable, and the insert is on the X chromosome. Line 3.1 is homozygous lethal, with the insert on the second chromosome, and is balanced over the *CyO* balancer. Line H4.1 is homozygous viable, with the insertion of the reporter construct on the second chromosome. All lines are available from the authors (8). Expression of the *yellow* marker is stable and apparently not subject to a position effect (see **Note 3**).

3.3. Design of the Genetic Crosses for the Functional Assay of I-Sce I Activity in the *Drosophila* Genome

The next step is the design of the actual genetic crosses. *Drosophila*, as a genetic system, offers a researcher a high degree of flexibility. However, there are several basic facts about *Drosophila* genetics to keep in mind while designing crosses. Genetic exchange under normal conditions in most laboratory strains is virtually absent in males. Several exceptions include hybrid dysgenesis induced by mobile elements (e.g., P element, hobo), chemical mutagens, radiation, and heat shock. This induced male recombination as

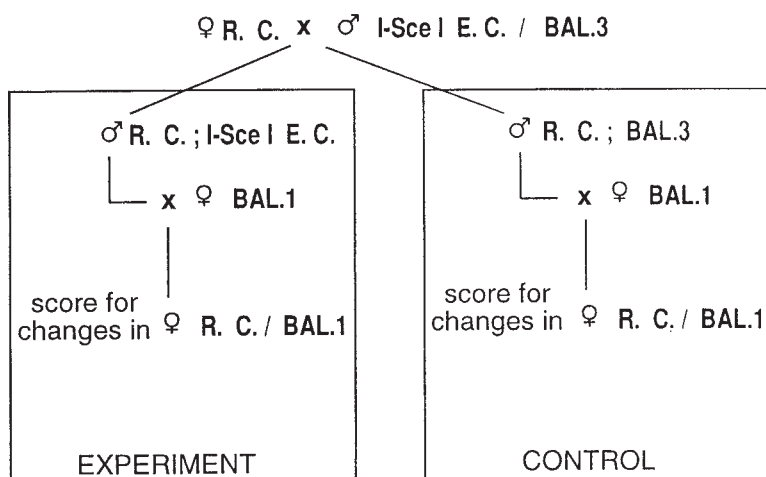


Fig. 2. General scheme of genetic crosses for the functional assay of I-Sce I activity in the *Drosophila* genome. R.C.—reporter construct. I-Sce I E.C.—expression construct. BAL.1 and BAL.3—balancers for the first and third chromosomes.

well as very low spontaneous male recombination ($<0.001\%$) is almost always mitotic in origin. Consequently, male recombination can be reduced to negligible levels by proper design of the crosses. In females, however, meiotic recombination is a normal event and occurs with high frequency. The general scheme of crosses is shown in **Fig. 2**. The main idea is to combine two constructs in one male genome: one construct supplying a functional protein, i.e., I-Sce I, the other being the reporter construct with the *yellow*⁺ phenotype which is easily scorable in the *yellow*[−] background. We found that depending on the location of the constructs in the genome, revertants to *yellow*[−] appear with a rate of 4–10% in the presence of functional I-Sce I enzyme. In the control crosses, where only the reporter construct, and no functional I-Sce I was present, no phenotypic revertants from *yellow*⁺ to *yellow*[−] were found (see **Note 4**).

4. Notes

1. In the construction of the expression vector, we made use of plasmids, generously provided to us by Maria Jasin (10) and Greg Donoho. The modified I-Sce I DNA sequence follows a nuclear localization signal (NLS in **Fig. 1A**).
2. A DNA sequence containing the $\beta 2$ -tubulin promoter was kindly provided to us by Renate Renkawitz-Pohl (12).
3. A distinguishing feature of the Y.E.S. marker (*yellow*, *enhancers* *suppressed*) is that it is insensitive to chromosomal position effects, confined between powerful

insulators, binding sites for the *su(Hw)* protein (13). We removed these insulators because binding of the *su(Hw)* protein close to the I-Sce I recognition site might interfere with the experiments.

4. Molecular characterization of the revertants (8) showed that introduction of a DSB by I-Sce I produced several types of alterations in the reporter construct. The first class of revertants retained only one perfect FRT sequence between two unchanged ends of the reporter construct (P element ends). The second type was represented by a reconstituted single perfect I-Sce I recognition site, which was found between two unchanged FRT sites. The third type of revertants was represented by sequences altered to various degrees found in place of the I-Sce I recognition site, along with two unchanged FRT sites. These products of the DSB repair events are consistent with the models proposed to explain the repair of DSBs induced by the P element transposase. However, some repair events seem to occur by the single-strand annealing pathway, leading to a loss of genetic information.

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Use of I-Sce I to Induce DNA Double-Strand Breaks in *Nicotiana*

Holger Puchta

1. Introduction

Double strand-breaks (DSBs) are key intermediates in DNA recombination reactions. The possibility of inducing DSBs at specific sites in the genome by the expression of rare-cutting endonucleases has resulted in a tremendous increase in our knowledge on the mechanisms of DSB repair, especially in yeast (**1**; see Chapter 32) and recently also in higher eukaryotes (**2**; see Chapter 37). In addition to its importance to the study of basic mechanisms of recombination, DSB induction leads to a dramatic enhancement of recombination frequencies and, therefore, has a great potential to be used as means for controlled genomic change. DSBs are repaired by two different recombination pathways: illegitimate or homologous. In somatic plant cells, homologous recombination is only used as a minor repair pathway. Consequently, an effective gene-targeting technique has not yet been established (**3**). However, DSB induction via the expression of I-Sce I or HO endonuclease increases homologous recombination frequencies one to two orders of magnitude for extrachromosomal (**4**) and intrachromosomal (**5**) recombination and for homologous integration (**6**). I-Sce I expression also induces homologous recombination between ectopic sites in the tobacco genome (H. Puchta, unpublished results). Synthesis-dependent strand annealing and one-sided invasion have been identified as the primary recombination mechanisms operating to repair DSBs in somatic plant cells (**6,7**). Recently, we have developed an assay using I-Sce I to study the repair of genomic DSBs by illegitimate recombination (**8**).

The basic principle of DSB-induced recombination studies is that in a controlled manner, at a given time-point, a site-specific endonuclease is expressed

in a transgenic plant line containing a recognition site of the enzyme integrated in the genome. Expression of the enzyme can be achieved either by the use of a specific promoter (e.g., heat-shock or meiotic tissue-specific) within a stably integrated transgene or via transient expression using a constitutive promoter. In principle, transient expression can be mediated either by various methods of direct gene transfer (e.g., polyethylene glycol-mediated transfection, electroporation, bombardment) or by the use of the soil bacterium *Agrobacterium tumefaciens* as transformation vector (for a detailed description of plant transformation techniques, see ref. 9).

This chapter describes an efficient method for transient expression of I-Sce I in tobacco (*Nicotiana tabacum*) seedlings via *Agrobacterium* (10), which is routinely used in our laboratory. In the protocol, site-specific integration is achieved by DSB induction. As depicted in Fig. 1, seedlings carrying the I-Sce I target site pTS in their genome are transformed by *Agrobacteria* harboring the repair construct pRC and the expression vector pCISceI. Homologous integration results in the restoration of a functional kanamycin resistance gene, which can be selected for during tissue culture.

2. Materials

1. *Nicotiana* seeds carrying a transgenic I-Sce I site in the genome (e.g., line 1–12, ref. 6).
2. *A. tumefaciens* strains carrying as binary vectors either the plant expression cassette of I-Sce I (pCISce I; see Note 1) or a homologous repair construct (pRC).
3. YEB medium: 5 g/L Bacto-beef extract, 1 g/L Bacto-yeast extract, 5 g/L peptone, 5 g/L sucrose, 0.493 g/L $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ sterilized by autoclaving.
4. 10 mM MgSO_4 solution, sterilized by autoclaving.
5. Murashige and Skoog (MS) medium (11; e.g., Sigma).
6. MS plates: MS solidified with 1% (w/v) agar.
7. Antibiotics stocks: 50 mg/mL kanamycin, dilute 1:2000 for bacteria and 1:100 for plants; 100 mg/mL rifampicin in dimethyl sulfoxide (DMSO), dilute 1:500; 100 mg/mL gentamicin, dilute 1:5000; 500 mg/mL cefotaxime, dilute 1:1000; 200 mg/mL vancomycin, dilute 1: 200.
8. Plant-specific compounds: 1 mg/mL 6-benzylaminopurine (BAP), dilute 1:200 for use; 1 mg/mL 1-naphthalene acetic acid (NAA), dilute 1:2000 for use.

3. Methods

3.1. Cocultivation of Transgenic Tobacco Seedlings with *A. tumefaciens* (see Note 2)

1. Put an ample amount of sterilized seeds (100–1000) of *N. tabacum* carrying an I-Sce I target site as transgene (e.g., line 1–12) on sterile filter paper soaked in

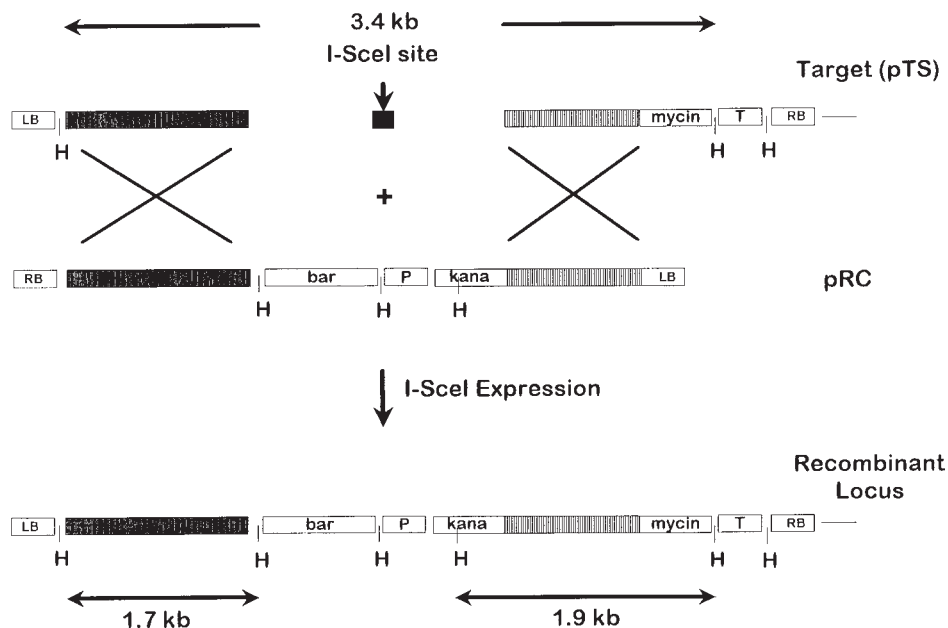


Fig. 1. Schematic representation of the recombination substrates used to detect homologous recombination after DSB induction. Maps of the DNA constructs used: pTS represents the target locus, drawn as T-DNA integrated into the plant chromosome. Between the homologous regions (shaded boxes) an I-Sce I site was inserted. pRC represents the repair construct, which carries homologies to both ends of the target locus. The homologous region within the kanamycin gene (shaded gray) is 1-kb in length, whereas the homology at the other end (dark shaded, hygromycin-specific sequences) is 1.3-kb in length. In cases of homologous recombination, recombinants should be obtained in which the kanamycin gene is restored and can thus be selected for. Whereas after Southern blotting of *Hind*III- (H) digested DNA, the original target locus should give a hybridization signal of 3.4-kb with a kanamycin-specific probe, homologous recombinants should show a 1.9-kb kanamycin-specific band and a 1.7-kb hygromycin-specific band. The *bar* gene in pRC is used as a selectable marker to calculate the absolute numbers of transformants in the experiments. LB, left border; RB, right border; P, cauliflower mosaic virus 35S promoter; T, cauliflower mosaic virus terminator.

sterile tap water in Petri dishes. Let them grow at 25°C for 1–2 wk on a 16-h light/8-h dark regime.

2. Inoculate an *Agrobacterium* strain carrying pCISceI in 20 mL of YEB medium containing the antibiotics rifampicin and gentamycin (see Notes 3 and 4). Inoculate another *Agrobacterium* strain carrying pRC in 20 mL of YEB medium containing the antibiotics rifampicin and kanamycin. Let them shake at 28°C overnight.

3. Harvest the cultures by centrifugation (10 min, 15,000g), wash the cells in 20 mL of MgSO_4 solution, and take them up in MgSO_4 solution to a final A_{600} of 0.5. Mix the two *Agrobacterium* strains pCISceI and pRC (4 vol:1 vol) (see **Note 5**).
4. Add in a 15-mL Falcon tube the seedlings to the bacterial solution, and expose them to reduced pressure (0.15 atm) in a sterile vacuum chamber for 10 min.
5. Distribute the seedlings on MS plates containing BAP and NAA, and cultivate them for 3 d in a growth chamber (25°C, 16-h light/8-h dark regime).

3.2. Selection of Cells in Which the DSB Has Been Repaired by Marker Restoration

1. Transfer the seedlings on MS plates containing BAP, NAA, kanamycin, vancomycin, and cefotaxime and put them back into the growth chamber.
2. Transfer the seedlings to new plates every week.
3. When calli arise, put them on new plates for propagation of clonal plant material.
4. From part of the callus material DNA can be extracted (**12**) to check via PCR or Southern blotting (**6**) if recombination has occurred (**Fig. 1**; see **Note 6**).
5. Cut shoots from the respective calli, and put them in Magenta boxes on MS medium containing kanamycin, vancomycin, and cefotaxime.
6. After root formation, the shoots can be transferred to soil to obtain flowering plants. After selfing, transgenic F_1 seeds can be harvested for genetic analyses.

4. Notes

1. Expression of the synthetic ORF of I-Sce I in higher eukaryotes will only work efficiently if the ORF is fused to a respective translation initiation site. In the case of pCISceI the respective region of ORF V of cauliflower mosaic virus is fused to the I-Sce I ORF (**4**).
2. The procedure described here relies on the high transformation efficiency of tobacco seedlings with *Agrobacterium*. Other plant species will have to be checked first in pilot experiments using marker genes (e.g., pBG5 as binary), concerning whether the same procedure can be used to obtain high transient expression and transformation rates. Other transformation methods (see **Subheading 1.**) might lead to better results.
3. To monitor the efficiency of the *Agrobacterium*-mediated transient expression of I-Sce I, a control transformation with a binary vector containing a transient (and selectable) marker gene (e.g., the β -glucuronidase gene) like pBG5 (**12**) is recommended.
4. Negative control transformations without expression of I-Sce I are indispensable to demonstrate that DSB induction is initiating the respective recombination event.
5. I-Sce I is stringent in cutting, but not in binding DNA (**14**). Therefore, efficient expression of the gene is needed to induce breaks. In coinoculations with a repair construct (e.g., pRC), *Agrobacteria* containing the I-Sce I gene have to be used in excess to obtain homologous integration. A 5- to 10-fold surplus has proven to be most efficient in our hands; at higher ratios, the amount of transferred repair construct was limiting in the integration reaction. However, the procedure will only work when high cotransformation efficiencies are achieved (see **Note 2**).

6. Homologous recombination does not always occur on both sides of the DSB, since in some kanamycin-resistant recombinants, the 1.7-kb hygromycin-specific fragment has been found to be absent (6). Moreover, recent experiments have revealed that a repair construct similar to pRC, but harboring homology only to the kanamycin-specific side of the DSB is almost as efficient in homologous integration as pRC. These results show that one-sided invasion plays a major role in homologous DSB repair in somatic plant cells, which is thus best described by the synthesis-dependent strand annealing and one-sided invasion models of recombination (7).

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Chromosomal Double-Strand Breaks Introduced in Mammalian Cells by Expression of I-Sce I Endonuclease

Christine Richardson, Beth Elliott, and Maria Jasin

1. Introduction

Until recently, investigators interested in analyzing the repair of chromosomal double-strand breaks (DSBs) in mammalian cells have been limited by the inability to introduce defined DSBs within the genome. Traditional methods of introducing breaks have included irradiation or the introduction of restriction enzymes (*1*; see Chapter 38); however, both of these methods cause multiple lesions at different chromosomal loci. Many of these types of studies have relied on cytogenetics for the detection of gross genomic changes owing to misrepair at these damaged sites.

To overcome the problem of examining the repair of specific DSBs in a complex mammalian genome, we have developed a system to introduce a single DSB at a known chromosomal locus using the bipartite I-Sce I endonuclease system. In this system, the recognition sequence for the rare-cutting I-Sce I endonuclease is introduced into a chosen chromosomal locus, and then an I-Sce I expression plasmid is introduced into cells; the produced I-Sce I endonuclease will cleave at its target site to result in a DSB (**Fig. 1**). The repair product of this DSB can then be analyzed by a number of assays, such as Southern blot analysis. In contrast to the cytogenetics that earlier studies often required, analysis of repaired I-Sce I breaks can include fine structure analysis of the DNA sequence around the break site.

I-Sce I is perhaps the best characterized of a class of endonucleases with long recognition sites that participate in intron-homing, a widely occurring phenomenon in lower eukaryotes, bacteria, and bacteriophage (*2*). Initially discovered during analysis of the ω genetic system of yeast mitochondria (*3,4*),

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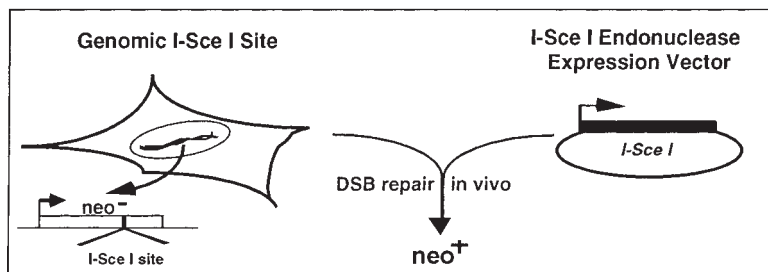
A**B**

Fig. 1. (A) The I-Sce I recognition sequence. (B) The I-Sce I endonuclease bipartite system.

I-Sce I recognizes an 18-bp nonpalindromic site (**Fig. 1A**; 5). Stringent specificity for this site is evident as mutational analysis has shown that most single basepair changes result in a partial if not severe decrease in cleavage by I-Sce I (6). Unlike I-Sce I, the specificity of many other intron-homing endonucleases may be limited because of their greater tolerance for base substitutions. Assuming a random organization of nucleotides, the frequency of an 18-bp site is one/ 7×10^{10} bp, which is only once in about 20 mammalian-size genomes. Although no endogenous I-Sce I recognition sites have been found in the yeast genome (7), no exhaustive search for such sites in mammalian organisms has been performed. Nevertheless, no deleterious effects of I-Sce I expression in several mouse cell lines and in transgenic mice have been observed (8–11). This nontoxicity coupled with the specificity of the enzyme has enabled us to exploit the I-Sce I system for the creation of specific DSBs within the mammalian genome.

The I-Sce I endonuclease acts to create a DSB within the genome that is then available for repair by the cell's DSB repair machinery. By contrast, site-specific recombinases, such as the lox-cre system, synapse two recognition sites and resolve intermediates in a precise and reversible manner that maintains one lox site in the genomic locus. Although this type of system has been used successfully for conditional gene knockouts, the concerted reaction does not allow for the study of DNA repair mechanisms or the specific roles that components of the DSB repair machinery play during the repair process (12).

Examination of a cell's ability to repair a DSB in the absence of one or more of these components will give insight into the role of individual components in

the DSB repair pathway. A number of rodent and human cell lines deficient in DNA repair components are available for these kinds of studies. In addition, there are cell lines derived from a number of genetic disorders, such as ataxia telangiectasia (AT) and xeroderma pigmentosum (XP), that demonstrate increased genomic instability, possibly owing to impaired DSB repair (13–15). Ku80, Ku70, and DNA-PK_{cs} are the three major subunits of the DNA-PK holoenzyme identified to participate in DSB repair; the Ku subunits are thought to be important in the protection of broken ends during this process (16–19). We have used an I-Sce I-induced DSB in wild-type and Ku80-deficient cells as an approach to examining repair without this end protection (20). We analyzed repair products in CHO-K1 hamster cells and their Ku80-deficient counterpart, xrs-6 cells, using a neomycin resistance (*neo*) gene disrupted by the I-Sce I recognition site. Within the *neo* gene, the I-Sce I site was flanked by a 4-bp duplication. This duplication can be used by the cell for nonhomologous end-joining in which short regions of terminal homology are sufficient to rejoin DNA ends, often producing deletions around the break site (9,21,22). In the CHO-K1 cells, this particular rejoining event was readily observed. In the xrs-6 cells, this rejoining event was not observed, demonstrating an impaired nonhomologous repair process (20). By contrast, repair from a homologous *neo* fragment in the Ku80-deficient cells is normal (20). These experiments with the I-Sce I system, therefore, are able to distinguish between a deficiency in homologous vs nonhomologous repair.

Investigators may also analyze the repair of a single I-Sce I break to identify homologous products that have been repaired by using different DNA templates, including endogenous homologous sequences, e.g., the second allele of a gene (23). Insertion of the I-Sce I recognition sequence into one of the two alleles of a gene has demonstrated a role for allelic conversion in DSB repair (23).

The I-Sce I system can also be used for more efficient gene targeting schemes. If a repair construct homologous to the DNA sequences around a chromosomal break site is provided at the time of I-Sce I expression, then homologous recombination events can be scored. We have reported that in mouse embryonic stem cells, a DSB enhances gene targeting at that site 50- to 1000-fold (8,24). Studies in mouse 3T3 and teratocarcinoma cells have demonstrated a similar increase (9–11). The chromosomal target site can be an artificially constructed locus or an endogenous locus. Several gene targeting strategies have used a “two-hit” strategy that requires recombination at a single site in the genome on two independent occasions; first, a selectable marker is inserted into a site in the genome, and second, the marker is removed to result in novel sequences at the locus not selectable by standard techniques (25–27). These types of “two-hit” protocols can be made more efficient by the inclusion of the I-Sce I system by introducing I-Sce I recognition sites during the first round of

targeting and using I-Sce I expression to induce a DSB at the target locus during the second round.

Given the great number of applications for targeted DSBs in the genome, the protocol below describes an I-Sce I gene targeting assay as a model system that can be easily modified for individual needs. In the first step, a construct containing the I-Sce I recognition site is stably inserted into the genome of a desired cell type. Insertion of the I-Sce I recognition site into the coding region of a selectable marker gene, such as the neo gene, will result in a neo null mutant with a G418-sensitive phenotype. Cotransfection of a second marker gene such as the hygromycin (hyg) gene allows for selection of clones that contain the mutant neo construct. In the second step, an I-Sce I expression plasmid is introduced into the cell for transient expression of the enzyme. Expression of I-Sce I will lead to a DSB at the target site. With cotransfection of a wild-type repair fragment homologous to the cut site in the genome, DSB-induced recombination will restore the wild-type neo gene. The frequency of these events can easily be scored by G418 resistance (G418^R). During gene targeting, the I-Sce I-induced homologous recombination event leads to complete loss of the cleavage site. No sequences from the recognition site will remain in the chromosome, and there is no possibility of the reverse reaction occurring. Following identification of the G418^R recombinants, investigators may choose to analyze further specific repair events by Southern blot, PCR, or sequencing assays.

2. Materials

2.1. Replacement of the Restriction Endonuclease NcoI Site with an I-Sce I Recognition Site in Neo Gene Coding Region

1. pMC1neo plasmid (28).
2. Double-stranded oligonucleotide linker containing the I-Sce I recognition sequence (**Fig. 1**) flanked by *NcoI* overhangs.
3. *NcoI* enzyme and buffer.
4. 3 M Sodium acetate, pH 5.2.
5. Ethanol, 100 and 80%.
6. T4 DNA ligase and buffer with ATP.
7. Competent bacterial strain, such as DH5 α .
8. LB agar plates supplemented with 50 μ g/mL ampicillin (Sigma, St. Louis, Mo).

2.2. Transfection of the Mutant Neo Construct into a Target Cell Line

1. Target cell line and appropriate growth medium.
2. Neo construct containing the I-Sce I recognition sequence (*see Subheading 2.1.*).
3. Hygromycin expression plasmid.
4. 0.2% Trypsin.
5. Hemacytometer.

6. Phosphate-buffered saline (PBS).
7. Gene Pulser cuvet, 0.4-cm electrode, gap = 50 (Bio-Rad #165-2088, Hercules, CA).
8. Bio-Rad Gene Pulser.
9. Hygromycin B (Boehringer, Mannheim, Germany).

2.3. Introduction of a Chromosomal DSB with the I-Sce I Expression Plasmid and Repair of the Break with a Homologous Neo Repair Vector

1. Target cell clone containing a single copy of the mutant neo sequence with the I-Sce I site and appropriate growth medium.
2. Circular I-Sce I expression plasmid.
3. Circular plasmid containing the Gene Targeting Neo Repair Fragment.
4. Items 4–8 of **Subheading 2.2**.
5. Geneticin (G418) (Gibco BRL, Gaithersburg, MD).

3. Methods

3.1. Replacement of the Restriction Endonuclease NcoI Site with an I-Sce I Recognition Site in Neo Gene Coding Region (see Note 1)

1. Using standard methods (29), harvest pMC1neo plasmid DNA, containing the coding region of the selectable marker gene driven by a promoter that will efficiently drive expression in the cell line of choice. Adjust the concentration to 1 µg/µL.
2. Digest 10 µg of pMC1neo plasmid with *NcoI* (see **Notes 2** and **3**) to create a single cut within the coding region of the neo gene. Combine in an Eppendorf tube: 10 µg of pMC1neo, 3 µL of 10X *NcoI* buffer, 10 U of *NcoI* enzyme and H₂O to a final volume of 30 µL. Incubate at 37°C for 1 h.
3. Purify the linearized DNA away from enzyme and buffer by ethanol precipitation (see **Note 4**). To the digestion reaction, add 3 µL of 3 M sodium acetate and 66 µL of 100% ethanol. Mix contents of the tube well. Sit in a dry ice-ethanol slurry for 10 min. Spin in a microcentrifuge at maximum speed (~15,000g) for 10 min. Wash once with 80% ethanol. Air-dry the DNA pellet, and resuspend in 20 µL of H₂O for a concentration of 0.5 µg/µL. By serial dilution, create a 100 ng/µL solution.
4. For the ligation mix, combine in an Eppendorf tube: 100 ng of *NcoI*-cleaved pMC1neo, 100 ng of I-Sce I linker with *NcoI* overhangs, 1 µL of 10X T4 DNA ligase buffer with ATP, 1 µL of T4 DNA ligase, and H₂O to a 10-µL total volume. Incubate at 16°C for 4–16 h (see **Notes 3** and **5**).
5. Transform bacteria (such as strain DH5α) by heat shock or by electroporation with half of the ligation reaction (29). Spread the resultant transformation reaction onto LB agar plates containing ampicillin. Incubate the plates at 37°C overnight, but not more than 16 h to avoid the appearance of satellite colonies.
6. Pick individual colonies, and grow overnight at 37°C in 10 mL of LB medium containing ampicillin (50 µg/mL).

7. Isolate plasmid DNA (29) to confirm the presence of the I-Sce I recognition site and the loss of the original *Nco*I site.
8. Once the correct plasmid is identified, a stock can be grown and sufficient DNA harvested for electroporation in the next step.

3.2. Transfection of the Mutant Neo Construct into a Target Cell Line

1. Expand the target cells in a 37°C, 5% CO₂ incubator to obtain a sufficient number for the transfection (see **Note 6**).
2. Change the medium 2 h prior to transfection. If the cells have been maintained in any selectable medium for other markers, then the medium used here should be nonselective. Return the cells to the 37°C, 5% CO₂ incubator for 2 h.
3. Remove the medium from the cells. Rinse each 10-cm plate of cells with 3 mL of PBS. Remove the PBS.
4. Trypsinize the cells for 8 min at 37°C using 2 mL of trypsin solution/each 10-cm plate of cells.
5. Wash the cells off the plates with nonselective medium, and transfer the cell suspension to a 15-mL conical tube.
6. Take an aliquot of cells for cell counting with a hemacytometer. Calculate the concentration and total number of cells.
7. Centrifuge the cell suspension at 1000 rpm (~160–200g) in table top Sorvall centrifuge for 10 min.
8. Aspirate the supernatant.
9. Resuspend the pellet in PBS to a cell concentration of 2×10^7 cells/mL.
10. Transfer 0.8 mL to an electroporation cuvet.
11. Add 60 µg of pMC1neo-I-Sce I mutant plasmid and 20 µg of pgk-hygromycin plasmid to the cuvet (see **Note 7**). Pipet several times to mix the solution without generating bubbles that may create an arc of current during the electroporation.
12. Electroporate the sample in a Bio-Rad Gene Pulser at 960 µF, 0.25 kV.
13. Dilute the sample with nonselective medium for a total volume of 2 mL.
14. Divide the sample onto 10-cm plates using necessary dilutions as determined for the cell line. Add 10 mL of nonselective medium to each plate. Incubate the plates overnight in a 37°C, 5% CO₂ incubator.
15. Wait 18–24 h postelectroporation, and then change the medium on the plates to medium supplemented with 150 µg/mL hygromycin B (see **Note 8**). At this time, one plate may be trypsinized and the cells counted to estimate the percentage of cell survival after electroporation; this number is helpful in determining the frequency of integration.
16. Hyg^R colonies can be identified and scored after 14 d.
17. Pick individual colonies and expand them. Continue to maintain the cells in hygromycin B.
18. Determine which hyg^R clones also contain a single copy of the desired pMC1neo-I-Sce I mutant construct by Southern blot analysis (29).

3.3. Introduction of a Chromosomal DSB with the I-Sce I Expression Plasmid and Repair of the Break with a Homologous Neo Repair Vector

1. Using standard methods (29), harvest an I-Sce I expression vector that contains the I-Sce I coding sequence driven by a promoter suitable for the target cell line.
2. Using standard methods (29), harvest the neo repair plasmid containing sequence homologous to the neo gene flanking the DSB site (*see Note 9*).
3. Expand one of the identified target colonies that contains a single copy of the pMCneo-I-Sce I mutant construct in a 37°C, 5% CO₂ incubator to obtain enough for the transfection (*see Note 6*).
4. Change the medium on the cells to nonselective medium 2 h prior to transfection. Return the cells to the incubator for 2 h.
5. Remove the medium from the cells. Rinse each 10-cm plate of cells with 3 mL of PBS. Remove the PBS.
6. Trypsinize the cells for 8 min at 37°C using 2 mL of trypsin/each 10-cm plate of cells.
7. Wash the cells off the plates with nonselective medium, and transfer the cell suspension to a 15-mL conical tube.
8. Take an aliquot of cells for cell counting with a hemacytometer. Calculate the concentration and total number of cells.
9. Centrifuge the cell suspension at 1000 rpm (~160–200g) in tabletop Sorvall centrifuge for 10 min.
10. Aspirate the supernatant.
11. Resuspend the pellet in PBS to obtain a cell concentration of 2×10^7 cells/mL.
12. Transfer 0.8 mL to an electroporation cuvet.
13. Add to the cuvet 25 µg of the I-Sce I expression plasmid and 25 µg of the neo repair construct (*see Note 10*). Pipet several times to mix the solution without generating bubbles.
14. Electroporate the sample in a Bio-Rad Gene Pulser at 960 µF, 0.25 kV.
15. Dilute the sample with nonselective medium for a total volume of 2 mL.
16. Divide the sample onto 10-cm plates using necessary dilutions as determined for the cell line. Add 10 mL of nonselective medium to each plate. Incubate the plates overnight in a 37°C, 5% CO₂ incubator.
17. Eighteen to 24 h postelectroporation, change the medium on the plates to medium supplemented with 200 µg/mL G418 (*see Note 8*).
18. Colonies can be identified and scored after 10–12 d (*see Note 11*).

4. Notes

1. Investigators should be aware that this basic protocol can be modified to insert the I-Sce I recognition site into any desired locus of the genome. Given the common use of neo vectors and the ease of G418 selection, we have found that disruption of the neo sequence with the I-Sce I recognition site and repair at this site to restore neo gene function is an efficient protocol that many investigators will find useful.

2. We have used this site to insert the I-Sce I recognition sequence, because it is a unique site in the plasmid and insertion here will result in a sufficient amount of neo coding sequence on either side of the break site for efficient crossing over with a homologous fragment during repair. However, any other unique site will also work.
3. With this protocol, there is a potential mechanism for the generation of Neo+ clones by a nonhomologous end-joining mechanism after I-Sce I cleavage because of a duplication of the *NcoI* overhangs (CATG) that is created during the construction. In this, the chromosomal ends may be processed to expose the microhomology at the CATG repeats at both ends. These ends can then anneal and be further processed to generate an *NcoI*+ Neo+ sequence. To eliminate the possibility of this type of end-joining reaction, mung bean nuclease treatment can be used to blunt the ends of the pMC1neo plasmid after *NcoI* digestion and prior to insertion of a modified linker with the I-Sce I recognition site.
4. We have also successfully used other standard DNA purification methods, such as the Qiaex DNA Extraction Kit (Qiaex, #20021).
5. We find that using a large excess of the linker obviates the need for a phosphatase reaction of the pMC1neo plasmid after digestion with *NcoI*. The use of linker oligonucleotides without a phosphate group at the ends will limit the reaction to one oligonucleotide insert/backbone. Sequencing of the final construct should be performed to determine the exact structure of the insertion.
6. In our hands, a greater transfection efficiency is obtained with cells growing in log phase at the time of electroporation. Therefore, we suggest that cells not be confluent on plates for the 24 h preceding electroporation.
7. The hygromycin plasmid may be substituted with any other selectable marker of choice. Certain promoters work more efficiently in different cell lines, and the type of cell line used should be a consideration when choosing both the selectable marker and the promoter to drive its transcription.
8. The optimal concentrations of hygromycin and G418 for selection may vary between cell lines. We recommend performing a survival curve on the cell line of choice to determine the best concentration for selection. Parental clones should all be G418-sensitive, since the pMC1neo has been mutated during cloning.
9. The homologous fragment should contain neo sequence flanking the *NcoI*-induced DSB site. Other investigators have reported that gene targeting requires a minimum of 500-bp of homology. However, given the strong induction of homologous recombination by I-Sce I, the required length of homology in these experiments may be shorter. We typically use fragments 700-bp long, with an approximately equal length on each side of the DSB. The tract of neo homology chosen should be constructed to ensure that random insertion of the neo repair plasmid downstream of a promoter region will not be sufficient to drive neo transcription and give G418 resistance.
10. Electroporation of a circular or linear targeting fragment may alter the types of repair events observed. For a perfect homologous event to occur, a linear fragment requires repair by a gene conversion pathway or a double crossover event (i.e., a two-sided event). However, with 3'-neo gene repair fragments, we have

also detected one-sided events that apparently use a homologous crossover on one side and nonhomologous repair on the other. In mouse embryonic stem cells, two-sided events appear to predominate (10), whereas in 3T3 cells, one-sided events are more common (9). In plants, both types of repair have been detected (30). Electroporation of a circular plasmid also may be repaired by a single crossover event if 3'-neo sequences are used. This mechanism results in the insertion of plasmid sequences into the genomic locus. If the neo repair sequence extends through the poly (A) region of the neo gene, then these single crossover events will be scored as G418^R, and identified by diagnostic RFLP digests and Southern blot analysis. However, if the neo repair sequence does not extend to the end of the coding region, i.e., is an internal neo gene fragment, then single crossover events will not be recovered in the final pool of clones.

11. The I-Sce I site has now been removed from the genome following recombination with the neo repair fragment.

Note Added in Proof

We have recently developed a PCR-directed assay to examine nonhomologous repair of I-Sce I double-strand breaks that do not result in *neo*+ cells. This protocol is detailed in ref. 31.

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Induction of DNA Double-Strand Breaks by Electroporation of Restriction Enzymes into Mammalian Cells

James P. Carney and William F. Morgan

1. Introduction

The introduction of restriction endonucleases into mammalian cells in culture provides a unique method for introducing double-strand breaks (DSBs) into the DNA of the host cell. Restriction enzymes recognize, bind, and cleave specific DNA sequences to produce a DNA DSB in the absence of other types of DNA damage. Not only is the DSB the only lesion produced in DNA, but the type of break (i.e., whether it has blunt ends or cohesive ends with 3'- or 5'-overhangs), the nucleotide sequences at the break site, and in certain instances, the precise chromosomal location of the induced break are also known. Once inside a cell, restriction enzymes are extremely efficient inducers of DSBs as measured by pulsed-field gel electrophoresis (1) and neutral filter elution (2). Consequently, the introduction of restriction enzymes into cells has been used to investigate the biological consequences of DSBs in the genome at the molecular, cytogenetic, and cellular level. At the molecular level, the rejoining of DSBs with known end structure has been investigated (3), and although it is not possible to determine the frequency of faithfully repaired DSBs, Phillips and Morgan (4) were able to describe unambiguously illegitimate recombination events occurring within the chromosome. At the cellular level, restriction enzyme-induced DSBs lead to mutations (5,6), chromosome rearrangements (7-10), gene amplification (11) and cell killing (5,10). Interestingly, restriction enzymes do not induce delayed chromosomal instability, suggesting that DSBs are not the signal responsible for initiating this process (12).

Restriction endonucleases can be introduced into cells in several ways, including permeabilization by Sendai virus or chemicals, trypsinization, osmotic

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shock, microinjection, electroporation, or after transfection of cells with expression vectors containing genes coding for an endonuclease (2,10,13–16). All of these methods work with varying efficiencies, and all have been demonstrated to produce chromosome aberrations and other cellular and cytological effects (2,10,13–16). Electroporation is a simple and convenient method of introducing restriction endonucleases into cells while providing efficiency of uptake approaching 100% (10).

This chapter describes the technique utilized in our laboratory for the introduction of restriction enzymes into Chinese hamster ovary (CHO) cells. Although described for use with CHO cells this technique is applicable to other mammalian cell lines. Additionally, a method for analysis of metaphase chromosomal aberrations that can be utilized to assay for the efficiency of enzyme electroporation is described.

2. Materials

1. Phosphate-buffered sucrose (PBS): 7 mM KH_2PO_4 , pH 7.4, 1 mM MgCl_2 , 272 mM sucrose. Store at 4°C; replace after 2 mo. Use commercially available stock solutions of MgCl_2 (see **Note 1**).
2. HEPES-buffered saline: 21 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 , 6 mM glucose. Store at 4°C; replace after 2 mo (see **Note 2**).
3. Culture medium: Chinese hamster ovary cells are cultured as monolayers in McCoy's 5A medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin. Human cells are cultured in RPMI 1640 medium with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin. Cells are cultured at 37°C in an atmosphere of 5% CO_2 in air.
4. Trypsin/EDTA stock solution: 0.5 g/L trypsin, 0.2 g/L EDTA, 1.0 g/L glucose, and 0.58 g/L NaHCO_3 in Puck's Saline A (Gibco BRL, Gaithersburg, MD). Store at –20°C.
5. Bio-Rad Gene Pulser or BRL Cell Porator (see **Note 3**).

3. Methods

3.1. Electroporation with the Bio-Rad Gene Pulser

1. Culture cells as described in the **item 3** of **Subheading 2**.
2. Make a cell suspension by treating a monolayer of exponentially growing cells with 5 mL of trypsin/EDTA. If working with suspension cultures, proceed to **step 4**.
3. Centrifuge for 5 min at 300g, and then discard the supernatant.
4. Wash the cell pellet, either in serum-free medium or in PBS, at ambient temperature or at 37°C. It is important to remove all trypsin/EDTA from the cells, because residual EDTA can inhibit restriction enzyme activity. Centrifuge the cells and resuspend them in PBS at ambient temperature to obtain a density of $1\text{--}10 \times 10^6$ cells/mL (see **Note 4**).

5. Put 800 μL of the cell suspension into a 0.4-cm electroporation cuvet, and add restriction enzyme or the appropriate restriction enzyme buffer without enzyme as a control. Mix by gently inverting the cuvet 10 times.
6. Electroporate the cells as soon as possible with 0.3 kV and 125- μF capacitance. Observe the time constant, which should be between 12 and 22 ms under these conditions.
7. Carefully remove the electroporated cells from the electroporation cuvet by using a clean, sterile Pasteur pipet. To avoid cell clumping, do not draw bubbles up into the cell suspension.
8. Add the suspension to a flask containing prewarmed medium (37°C).
9. Replace the medium with fresh medium after 1.5–2 h to eliminate any unattached cells (17). This step is very important. In addition to removing any dead, dying, or unattached cells, it also removes the residual electroporation buffer from the cell-culture medium.

3.2. Electroporation with the BRL Cell Porator

1. Make a cell suspension by treating a monolayer of exponentially growing cells with 5 mL of trypsin/EDTA. If working with suspension cultures, proceed to **step 3**.
2. Centrifuge for 5 min at 300g, and then discard the supernatant.
3. Wash the cell pellet, either in serum-free medium or in HEPES-buffered saline, at 4°C.
4. Centrifuge the cells and resuspend them in HEPES-buffered saline at 4°C to obtain a density of 2×10^6 cells/mL.
5. Put 800 μL of the cell suspension into a 0.4-cm electroporation cuvet, and add restriction enzyme or the appropriate restriction enzyme buffer without enzyme as a control. Mix by gently inverting the cuvet 10 times.
6. Electroporate the cells under the following conditions: field strength of 650 V/cm (indicated voltage of 260 divided by 0.4-cm electrode gap), capacitance of 1600 μF , and the electroporator set at low resistance.
7. Immediately incubate the cells on ice for 5 min (*see Note 5*).
8. Carefully remove the cell suspension from the cuvet, and mix with 10 mL of medium in a tissue-culture dish.
9. Incubate the cells at their optimal growth temperature for 1 h, and then disperse the cell clumps by pipeting up and down.
10. Incubate cells for 1 h, and then replace the medium with fresh medium.

3.3. Metaphase Cell Chromosome Aberration Assay (see Note 6)

This section is included as a method to confirm that the restriction enzyme has entered the cell nucleus and cleaved chromosomal DNA. Restriction enzymes cleave cellular DNA in a cell cycle-independent manner to produce both chromosome (G_1) type aberrations and chromatid (S/G_2) aberrations (10,18). Consequently, analysis of metaphase chromosome aberrations provides proof of enzyme activity in the nucleus. Winegar and Lutze (19) demonstrated a very

high frequency of uptake of β -galactosidase into cells using a range of electroporation conditions (*see Note 7*). However, when using their electroporation conditions to introduce restriction enzymes into cells, only some of the electroporation parameters resulted in chromosomal aberrations. This suggests that not all electroporation conditions permeabilize both the cell and nuclear membranes. Analysis of the frequency of metaphase cells showing chromosomal abnormalities also provides some idea of the *minimum* number of cells incorporating enzyme and, thus, the efficiency of electroporation (*20*).

1. At various times after electroporation, add Colcemid to exponentially growing cells at a final concentration of $2 \times 10^{-7} M$.
2. Collect metaphase cells after 1–3 h by gently shaking the flask.
3. Centrifuge the metaphase cells at approx 300g, and resuspend them in hypotonic 75 mM KCl prewarmed to 37°C.
4. Incubate the cells at 37°C for 5–15 min, then centrifuge, and resuspend them in methanol for 2 min.
5. Centrifuge the cells, and resuspend them in methanol-acetic acid (3:1) for 5 min.
6. Centrifuge the cells, and resuspend them in a small volume of methanol-acetic acid.
7. Using a pipet, drop the metaphase cells onto clean glass microscope slides, and allow them to air-dry.
8. Stain the slides with 4% Giemsa for 2–5 min, dry, and permanently mount them with a cover slip for microscopic analysis of cytogenetic abnormalities.

4. Notes

1. For optimal efficiency of restriction enzyme uptake after electroporation with the Bio-Rad Gene Pulser, we use phosphate-buffered sucrose as the electroporation medium. For reasons we do not understand, it is best to prepare this solution using a commercially supplied stock solution of $MgCl_2$ (e.g., 1 M concentration; Sigma, St. Louis, MO). Cell-culture medium without serum supplements can also be used.
2. For optimal efficiency of restriction enzyme uptake after electroporation with the BRL Cell Porator, we use HEPES-buffered saline as the electroporation medium.
3. Both the Bio-Rad Gene Pulser and the BRL Cell Porator are effective for the introduction of enzymes into cells, but the most effective conditions for the BRL machine also result in relatively high levels of cell death (*10*). Consequently, we prefer to use the Bio-Rad Gene Pulser, because we achieve efficient uptake of the restriction enzyme with very little electroporation-induced cell death.
4. We generally use 2×10^6 cells/mL, although this system works efficiently with up to 10^7 cells/mL.
5. In our experience, incubating the cells on ice for 5 min immediately following electroporation increases both cell survival and the efficiency of enzyme uptake when the BRL electroporation apparatus is used. However, this step has no noticeable effect when the Bio-Rad Gene Pulser is used.

6. The metaphase cell chromosome aberration assay is included as one method available for confirming restriction enzyme action in the cell nucleus. It is possible to use cell kill as an assay for deciding on the appropriate amount of enzyme to be used.
7. Electroporation can be used to introduce a variety of proteins into cells, including monoclonal antibodies (21,22), functional enzymes (23), other endonucleases in addition to restriction enzymes (24), and a host of other proteins and protein products (25–27).

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In Vitro Rejoining of Double-Strand Breaks in Genomic DNA

George Iliakis and Nge Cheong

1. Introduction

A large number of studies suggests that double-strand breaks (DSBs) induced in DNA by ionizing radiation or chemical agents are critical lesions, which if unrepaired or misrepaired may kill a cell, or cause its transformation to a cancer cell. Cells have developed efficient repair mechanisms to remove DSBs and restore integrity to their DNA. Despite the potential importance of DSBs in cell killing and transformation, relatively little is known regarding DSB repair mechanisms and the enzymes involved. However, characterization of these processes is crucial for a complete understanding of the consequences of exposure to agents, inducing DSBs.

Although homologous recombination appears to be the preferred mechanism by which DSBs are repaired in lower eukaryotes, such as yeast, nonhomologous end-joining appears to be the preferred mechanism in higher eukaryotes, and although the DNA-dependent protein kinase has recently been directly implicated in the rejoining of DSBs in mammalian cells, the actual mechanism(s) of this rejoining remains unknown (for a recent review, *see I*). In addition, since mammalian cells appear to utilize multiple pathways for nonhomologous end-joining, a rather complex enzymology and regulation remain to be elucidated. Study of these pathways will likely require a combination of biochemical and genetic techniques. Because the generation of mutants is an arduous task in mammalian cells, biochemical studies based on in vitro assays become increasingly important. Such studies may not only allow the functional characterization of factors identified through genetic studies, but they may also allow the identification and characterization of products of genes whose mutation is lethal to the cell.

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This line of argument has led to the development of *in vitro* assays for DSB rejoining using plasmid DNA as a substrate linearized with restriction endonucleases to generate various combinations of ends (e.g., 2–6, see Chapter 40). These assays are very useful and have generated information essential for our understanding of the mechanisms utilized by the cell to rejoin DNA ends. However, because of differences in the organization and sequence of genomic and plasmid DNA, and because multiple pathways are available for end-joining in mammalian cells, it is possible that different mechanisms are preferred for rejoining DSBs induced in plasmid vs genomic DNA. For this reason, we developed and describe here an *in vitro* assay that allows the study of DSB rejoining in genomic DNA. The assay utilizes as a substrate DSBs induced by various means (e.g., ionizing radiation, restriction endonucleases, bleomycin, and so forth) in genomic DNA prepared from agarose-embedded cells after appropriate lysis. At present, two extremes in terms of the state of DNA organization have been tested: “Naked” DNA (7–9) and DNA organized in chromatin. The former state is generated by complete lysis of cells embedded in agarose using detergents and proteases, but the latter state is generated by gentle lysis that leaves nuclei intact. Although not yet fully tested, intermediate states of chromatin organization can also be generated by gradually extracting nuclear proteins and histones with increasing salt concentrations.

The unique feature of the assay lies in the fact that the agarose fiber network that encapsulates the intact cell generates a “cage” that protects and restricts the expansion and mobility of the cellular DNA, or chromatin, particularly when freed upon lysis from the nucleus. This cage is expected to preserve essential features of DNA organization, especially after treatment to induce DSBs, and also to prevent extensive entangling. In this way, elements of the *in vivo* DNA repair process are maintained, including the large-mol-wt DNA, the low number of DSBs (ends) present/Mbp, the high local DNA concentration, the sequence context, and for nuclei-based or derivative assays, the possibility of retaining elements of chromatin structure.

Here are described the protocols developed to carry out these *in vitro* reactions for DSB rejoining. The required procedures include:

1. Preparation of HeLa-cell extract
2. Preparation of the DNA (naked DNA or nuclei).
3. Generation of DSBs in the DNA.
4. Assembly of *in vitro* repair reactions.
5. Assay for DNA damage using pulsed-field gel electrophoresis.

Figure 1 outlines these steps.

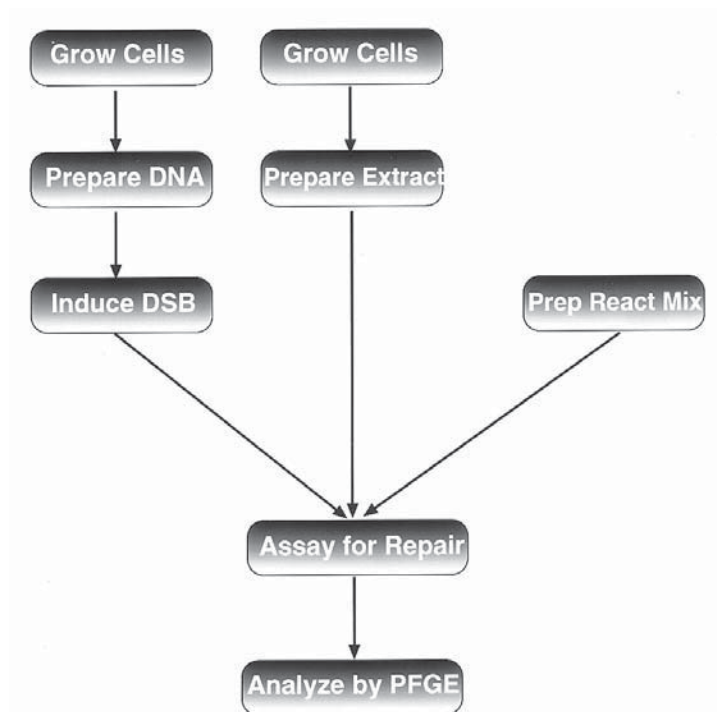


Fig. 1. Outline of the steps required to analyze repair of genomic DSBs in vitro.

2. Materials

2.1. Preparation of HeLa-Cell Extract (see Note 1)

1. Minimum Essential Medium (MEM) modified for suspension cultures (S-MEM), supplemented with 5% iron supplemented bovine serum and antibiotics (penicillin 100 U/mL, streptomycin 100 µg/mL).
2. Hypotonic buffer solution: 10 mM HEPES, pH 7.5 at 4°C (stock 0.6 M, pH 7.5 at room temperature), 1.5 mM MgCl₂ (stock 0.5 M), 5 mM KCl (stock 2 M). Immediately before use, add 0.2 mM phenylmethylsulfonyl fluoride (PMSF) (stock 100 mM in isopropanol; can be stored at -20°C for more than a month), and 0.5 mM dithiothreitol (DTT) (stock 1 M in H₂O; store at -20°C).
3. High-salt buffer: 10 mM HEPES, pH 7.5 at 4°C, 1.4 M KCl, and 1.5 mM MgCl₂.
4. Dialysis buffer: 25 mM Tris-HCl, pH 7.5 at 4°C (stock 1 M, pH 7.5 at 4°C), 10% glycerol, 50 mM NaCl (stock 5 M), 1 mM EDTA (stock 0.5 M, pH 8.0). Immediately before use add 0.2 mM PMSF and 0.5 mM DTT.
5. Microcarrier spinner flasks of 30 L nominal volume (Bellco Glass Inc.)
6. Microcarrier magnetic stirrers (Bellco Glass Inc.)
7. Tissue-culture dishes (100 mm).
8. Dounce homogenizer with B pestle, 50 mL.

2.2. Preparation of Naked DNA from Agarose-Embedded Cells

1. McCoy's 5A growth medium (Gibco BRL) supplemented with 5% bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin.
2. HEPES-buffered McCoy's 5A growth medium. Prepared from McCoy's 5A medium by reducing the NaHCO₃ concentration to 5 mM and by adding HEPES to a final concentration of 20 mM.
3. ¹⁴C-thymidine specific activity ~50 mCi/mmol (*see Note 2*).
4. 500 µM thymidine in water.
5. 1% solution of InCert agarose (FMC) in serum-free HEPES-buffered McCoy's 5A growth medium (no serum added). Incubate at 42°C in a water bath to prevent solidification.
6. 3.5-mm diameter glass tubes cut in 15- to 20-cm lengths. Seal one end with tape. Precool by immersing in ice.
7. Lysis solution: 10 mM Tris-HCl, pH 7.5 at 50°C, 100 mM EDTA, 50 mM NaCl, 1% N-lauryl sarcosyl (NLS; stock is 10% w/v in H₂O). Add 0.2 mg/mL Proteinase (Sigma, cat. no. P6911, St. Louis, MO) just before use.
8. Washing buffer: 10 mM Tris-HCl, pH 7.5, at 4°C, 100 mM EDTA and 50 mM NaCl.
9. RNase solution: 0.1 mg/mL RNase A in 10 mM Tris-HCl, pH 7.5, at 37°C, 100 mM EDTA, 50 mM NaCl.

2.3. Preparation of Nuclei from Agarose-Embedded Cells

1. Saponin lysis solution: 50 mM Tris-HCl, pH 8.0, at room temperature, 100 mM EDTA, 100 mM KCl, 0.05% saponin, 10% glycerol. Add 200 mM β-mercaptoethanol immediately before use.
2. Nuclei washing solution: 10 mM Tris-HCl, pH 8.0, at room temperature, 100 mM EDTA. Add 1 mM PMSF immediately before use.
3. Nuclei prereaction washing solution: 10 mM Tris-HCl, pH 8.0 at room temperature. Add 1 mM PMSF just before use.

2.4. Generation of DSBs in DNA

The requirements will vary depending on the experimental protocol. *See Subheading 3.4.* for details.

2.5. Assembly of In Vitro Repair Reactions: Naked DNA

1. Prereaction washing buffer: 40 mM HEPES-KOH, pH 7.4, at 37°C, 1 mM EDTA, 20 mM KCl, 3 mM MgCl₂.
2. Reaction buffer: 40 mM HEPES-KOH, pH 7.4, at 37°C, 20 mM KCl, 3 mM MgCl₂, 10 µM (each) dNTPs (stock 2 mM each), 1.5 mM ATP (stock 100 mM), 1 mM β-mercaptoethanol. To assemble the reactions, prepare a 10X solution (*see Note 3*). Dilute with water to the final volume. Account for the volume of the extract (and of other components that may be added in the reaction) by reducing proportionally the amount of water added.
3. Lysis solution: 10 mM Tris-HCl, pH 7.5, at 50°C, 100 mM EDTA, 50 mM NaCl, 2% NLS, 0.1 mg/mL proteinase.

4. Washing solution: 10 mM Tris-HCl, pH 7.5, at 37°C, 100 mM EDTA, 50 mM NaCl.
5. RNase treatment solution: 0.1 mg/mL RNase in 10 mM Tris-HCl, pH 7.5, at 37°C, 100 mM EDTA, 50 mM NaCl.

2.6. Assembly of In Vitro Repair Reactions: Nuclei

1. Nuclei prereaction buffer: 20 mM Tris-HCl, pH 7.5 at room temperature, 2 mM EDTA, 50 mM KCl, 5 mM MgCl₂.
2. Nuclei reaction buffer: 20 mM Tris-HCl, pH 7.5, at room temperature, 2 mM EDTA, 50 mM KCl, 5 mM MgCl₂, 10 μM dNTPs, 1.5 mM ATP, 1 mM β-mercaptoethanol. *See item 2 of Subheading 2.5.* for details in the use of this solution to assemble reactions.
3. Lysis solution: 10 mM Tris-HCl, pH 8.0 at room temperature, 100 mM EDTA, 2% NLS, 0.1 mg/mL proteinase.
4. Washing solution: 10 mM Tris-HCl, pH 8.0 at room temperature, 100 mM EDTA, 50 mM NaCl.
5. RNase treatment solution: 0.1 mg/mL RNase in 10 mM Tris-HCl, pH 7.5, at 37°C, 100 mM EDTA, 50 mM NaCl.

2.7. Pulsed-Field Gel Electrophoresis

1. 0.5X TBE: 45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA, pH 8.2. Prepare a 5X stock solution.
2. Gel dryer and model 583 gel dryer filter paper, 34 × 45 cm² (Bio-Rad, Hercules, CA).
3. PhosphorImager.
4. For Asymmetric Field Inversion Gel Electrophoresis (AFIGE): Horizontal gel electrophoresis system, model H4, 20-well comb (3.5 × 6 mm²) (Gibco BRL, Grand Island, NY); refrigerated water bath and circulating pump; GTC agarose (FMC, Rockland, ME).
5. For Clamped Homogeneous Electric Field (CHEF) gel electrophoresis: CHEF DRII apparatus (Bio-Rad); refrigerated water bath and circulating pump; Seakem agarose (FMC).

3. Methods

3.1. Preparation of Cell Extract (see Note 4)

The method described here allows the preparation of cell extract from 10 L of cell suspension. Higher or lower amounts of extract can be prepared by appropriate scaling.

1. Grow HeLa cells at 37°C for 3 d in 25 100-mm tissue-culture dishes prepared at an initial density of 6×10^6 cells per dish in 20 mL of S-MEM supplemented with serum and antibiotics. The final density after 3 d of growth should be $\sim 20 \times 10^6$ cells/dish, giving a total of 5×10^8 cells in 25 dishes.
2. Trypsinize the cells from all dishes, and resuspend in 10 L of prewarmed complete growth medium in a 30 L nominal volume microcarrier flask, thoroughly

pregassed with 5% CO₂ in air. The initial cell concentration should be $\sim 5 \times 10^4$ cells/mL. Place in a warm room at 37°C; provide adequate stirring (~ 60 rpm).

3. Allow the cells to grow for 4 d, to a final concentration of $4\text{--}6 \times 10^5$ cells/mL. Do not exceed this concentration of cells.
4. Collect the cells by centrifugation (7 min at 2500g). Collection should be fast and is best done using a refrigerated centrifuge that can accept 1-L bottles (e.g., Beckman J6-MI, Fullerton, CA). All further processing should be carried out at 0–4°C.
5. Rinse twice in PBS and centrifuge (5 min at 500g). Determine the packed cell volume (PCV) (~ 12 mL total).
6. Resuspend the cell pellet in 5 PCVs of hypotonic buffer solution, and centrifuge quickly (10 min, 1200g). Cells swell, and the PCV approximately doubles.
7. Determine the new PCV. Resuspend the cell pellet in an equal volume of hypotonic buffer, and disrupt in a Dounce homogenizer (20 strokes, pestle B). It is advisable to test cell disruption using a phase-contrast microscope.
8. Add 0.11 vol of high-salt buffer, and centrifuge at 3000g for 20 min.
9. Carefully remove the supernatant, and centrifuge at 100,000g for 1 h.
10. Place the resulting extract (S100) in dialysis tubing with a mol-wt cutoff of 10–14 kDa, and dialyze overnight against 50–100 vol of dialysis buffer.
11. Collect the extract. Centrifuge at 10,000g for 20 min to remove precipitated protein. Aliquot and snap-freeze. Store at -70°C . Keep a small aliquot for determining protein concentration using the Bradford assay (Protein Assay kit, Bio-Rad).

3.2. Preparation of Naked DNA from Agarose-Embedded Cells

The procedure described here produces naked DNA from agarose-embedded cells with no detectable impurities of protein and RNA. The protocol is for the preparation of DNA from the human lung carcinoma cell line A549, but it can be modified to prepare DNA from other cell lines (*see Note 5*).

1. Plate 2×10^6 A549 cells/100-mm dish for 6 d in 20 mL of McCoy's 5A supplemented with serum, antibiotics, as well as with $0.01 \mu\text{Ci/mL}$ ^{14}C -thymidine (methyl- ^{14}C -thymidine) and $2.5 \mu\text{M}$ cold thymidine (*see Note 2*). Cells reach a plateau after this period of growth, with more than 95% accumulated in G₁/G₀ phase (if possible verify by flow cytometry). There will be $\sim 2 \times 10^7$ cells/dish.
2. Trypsinize the cells, collect by centrifugation (5 min at 500g), and wash once in serum-free HEPES-buffered McCoy's 5A growth medium.
3. Resuspend the cells in serum-free medium at a concentration of 6×10^6 cells/mL. Make sure no clumps are present at this stage. If clumps are visible under the microscope, they should be carefully disrupted using a narrow-bore Pasteur pipet, or a syringe with a 19-gauge needle.
4. Mix with an equal volume of 1% InCert agarose. The final cell concentration is 3×10^6 cells/mL, and the final agarose concentration is 0.5%.
5. Quickly pipet the suspension into precooled 3.5-mm diameter glass tubes and incubate in ice until solidification (2–5 min).

6. Gently remove the solidified agarose from the tubes and cut 5-mm long blocks (~50 μL per block; $\sim 1.4 \times 10^5$ cells/block; $\sim 1.0 \mu\text{g}$ DNA per block, assuming 5 pg DNA/diploid human G_1 cell—A549 cells have 1.4-fold more DNA than human diploid cells; the local DNA concentration in the “cage” generated by a cell of $\sim 15 \mu\text{m}$ in diameter is $\sim 4 \text{ mg/mL}$). This operation should be carried out in the cold room. Immerse the agarose blocks into an excess of serum-free HEPES-buffered McCoy’s 5A growth medium, enough to cover them completely.
7. Transfer the agarose blocks to lysis solution (5 blocks/mL), and incubate at 4°C for 45 min.
8. Transfer to 50°C , and incubate for 12 h in a moderately shaking water bath (longer incubation is also possible if desired or if more convenient).
9. Carefully remove the lysis solution, and rinse once in washing solution (5 blocks/mL).
10. Remove the washing solution, add fresh washing solution, and repeat the washing for a further 6 h with three changes in a cold room with moderate shaking using an orbital shaker.
11. Carefully remove the washing solution, and treat with RNase for 1 h at 37°C in a moderately shaking water bath (5 blocks/mL).
12. Rinse the agarose blocks in washing solution, and transfer to fresh washing solution. Incubate in washing solution at 4°C for four more hours under continuous shaking. Change the washing solution every 2 h.
13. Transfer the agarose blocks into fresh lysis solution, and incubate at 50°C for 12 h in a moderately shaking water bath. (Proteinase concentration can be reduced to 0.1 mg/mL at this stage.)
14. Wash as described in **steps 9–11**. After completion of these steps, RNA and protein are not detectable in the agarose blocks. The agarose blocks are now ready to be used for in vitro repair reactions; they can be stored in washing buffer at 4°C in the dark for at least 12 mo (*see* **Note 6**).

3.3. Preparation of Nuclei from Agarose-Embedded Cells

1. Grow A549 cells to a plateau phase as described in **step 1** of **Subheading 3.2**. (*see* **Note 5**).
2. Trypsinize, wash, and embed the cells in agarose as in **steps 2–6** of **Subheading 3.2**.
3. Carefully transfer the agarose blocks to saponin lysis solution, and incubate for 45 min at 4°C ; then transfer to 50°C for 2 h in a moderately shaking water bath.
4. Remove the lysis solution, and rinse the agarose blocks in nuclei washing solution.
5. Add fresh nuclei washing solution, and incubate for 1 h at 37°C in a shaking water bath under moderate shaking. Repeat the washing procedure, if necessary. At this stage, the agarose blocks are nearly free of cytoplasm and are ready to be used in repair reactions. Agarose blocks so treated should be used immediately. If necessary, they can be stored overnight at 4°C . Longer storage is not advisable, but if adopted, it should be tested first.

3.4. Generation of DSBs in the DNA

DSBs can be induced, either in naked DNA or in nuclei embedded in agarose, by different agents. The choice of agent will be determined by the studies to be performed. Ionizing radiation can be used to induce DSBs randomly with nonligatable ends. If ligatable ends are desired, treatment with rare-cutting restriction endonucleases can be utilized. Aspects of these treatments are described next.

Exposure to X-rays or γ -rays can be carried out after embedding cells in agarose as described above in **steps 1–6 of Subheading 3.2.**, and before lysis to generate naked DNA or nuclei. Doses in the range of 20–40 Gy should be used with the detection assays outlined here. Alternative detection assays should be utilized if experiments requiring lower doses must be performed. When intact, G₁-phase, A549 cells are exposed to radiation, 36 DSBs are produced/cell/Gy of 250 kVp X-rays (2-mm Al filter). Radiation exposure is carried out with the agarose blocks immersed in serum-free HEPES-buffered McCoy's 5A medium on ice. After irradiation, the agarose blocks are lysed for the preparation of either nuclei or naked DNA, as outlined in the corresponding protocols.

When experiments are performed with naked DNA, it is more convenient to expose the agarose blocks to radiation after completion of the lysis procedure, shortly before the actual experiment (in prereaction buffer). Because naked DNA is more sensitive to breakage by ionizing radiation than DNA organized in chromatin, approximately threefold lower doses should be used (5–15 Gy) in order to generate DSBs equivalent in number to that produced in intact cells exposed to doses of 10–50 Gy (8).

When rare-cutting restriction endonucleases are used to generate DSBs digestions should be carried out in the buffer recommended by the supplier of the enzyme (frequently cutting restriction endonucleases generates extensive DNA fragmentation that is only partly amenable to ligation under the conditions of the assay). It is advisable to wash the plugs in the enzyme buffer for 2–4 h with at least one change before proceeding with the actual digestion. The agarose barrier makes it necessary to increase the units of enzyme, as well as the treatment time employed. Increasing the treatment time to 4 h and the enzyme activity units 5- to 10-fold above what would be used to digest DNA in solution provide, in general, satisfactory results.

3.5. Assembly of In Vitro Repair Reactions: Naked DNA

Once cellular extracts and agarose blocks with DSBs are prepared, the in vitro reactions to assay rejoining can be assembled.

1. Wash the required number of plugs in prereaction buffer (5 agarose blocks/mL) for 2 h at 4°C under continuous, moderate shaking using an orbital shaker. Repeat the washing procedure two to three times.

2. Transfer the agarose blocks (1/50 μ L reaction) to 1.5-mL Eppendorf tubes containing 50 μ L (total volume) of reaction buffer supplemented with cell extract (50–100 μ g/reaction). Maintain the reactions at 4°C during these preparation steps.
3. Incubate at 37°C in a shaking water bath for various periods of time, as required by the experimental protocol.
4. After the required incubation period for repair, transfer the agarose blocks into cold lysis solution (0.5 mL/agarose block, in 10-mL round-bottom tubes) for 30 min and then to 50°C for 2 h in a moderately shaking water bath.
5. Wash the agarose blocks for 1 h at 37°C in washing buffer (2.5 mL/agarose block). Remove the washing buffer, and treat with RNase A solution (0.5 mL/agarose block) for 1 h at 37°C in a moderately shaking water bath.
6. Remove the RNase solution, and wash the agarose blocks in washing buffer for 2 h at 4°C in a moderately shaking water bath (optional). Repeat the wash step with fresh washing buffer (optional). At this point, the agarose blocks are ready to be loaded for pulsed-field gel electrophoresis.

3.6. Assembly of In Vitro Repair Reactions: Nuclei

1. Wash the required number of plugs in nuclei prereaction buffer for 2 h at 4°C.
2. Transfer the agarose blocks to 1.5-mL Eppendorf tubes containing 50 μ L of nuclei reaction buffer supplemented with cell extract (50–100 μ g/reaction). Keep the reactions at 4°C during these preparation steps.
3. Incubate at 37°C for various periods of time, as required by the experimental protocol.
4. After the incubation period for repair, transfer agarose blocks into 1 mL of cold lysis solution (in 10-mL round-bottom tubes) for 30 min and then to 50°C for 12 h in a moderately shaking water bath.
5. Wash the agarose blocks for 1 h at 37°C in washing buffer. Repeat the wash step with fresh washing buffer. Remove the washing buffer, and treat with 1 mL RNase A solution for 1 h at 37°C in a moderately shaking water bath.
6. Remove the RNase solution, and wash the agarose blocks in washing buffer for 2 h at 4°C in a moderately shaking water bath (optional). Repeat the wash step with fresh washing buffer (optional). At this point, the agarose blocks are ready to be loaded for pulsed-field gel electrophoresis.

3.7. Assay for DNA Damage Using Pulsed-Field Gel Electrophoresis

A variety of pulsed-field gel electrophoresis techniques have been developed to quantitate DSBs in cellular DNA. Any one of these methods can, in principle, be used to evaluate in vitro repair in the reactions described above. The choice will depend on equipment availability and the requirements of the individual experiment. We routinely use AFIGE when a large number of samples needs to be evaluated and precise sizing of DNA is not required. When sizing of DNA fragments is considered important, we use CHEF gel electrophoresis (8). A detailed description of these methods is beyond the scope of

this protocol, and only the parameters routinely used in our laboratory for the quantitation of DSBs using the above assays are presented next.

3.7.1. Asymmetric Field Gel Electrophoresis (AFIGE)

1. Cast a 0.5% GTC agarose gel (250 mL, $20 \times 25 \times 0.5 \text{ cm}^3$) with 0.5 $\mu\text{g/mL}$ ethidium bromide in 0.5X TBE using the appropriate combs. Allow the gel to solidify at room temperature, and transfer to 4°C for 1 h for further solidification. Remove the combs just before loading.
2. Load the gel by inserting the agarose blocks into the wells. Close the wells with 0.5% agarose.
3. Place the gel into the electrophoresis box containing 2.5 L of precooled (10°C) 0.5X TBE. Cooling is achieved by a refrigerated water bath and a circulating pump.
4. Run at 10°C for 40 h by applying cycles of 1.25 V/cm (50 V) for 900 s in the direction of DNA migration and 5.0 V/cm (200 V) for 75 s in the reverse direction.
5. After completion of electrophoresis, place the gel under a UV table, and take a photograph for documentation.
6. Dry the gel by carefully placing it on filter paper in a gel dryer (1 h at 80°C), under vacuum (20–25 mmHg).
7. Expose a PhosphorImager screen to the dried gel for 24–48 h.
8. Quantitate the DSBs present in the samples by evaluating the amount of activity present in the entire sample over that present in the lane (fraction of activity released; FAR). This parameter is directly related to the number of DSBs present in the DNA.
9. Plot the results as a function of time. A typical gel and its quantitation are shown in **Fig. 2**.

3.7.2. CHEF Gel Electrophoresis

CHEF gel electrophoresis is performed in a CHEF DRII apparatus using a 0.8% Seakem agarose gel in 0.5X TBE at 10°C for 68 h at 45 V with 60-min pulses. After completion of electrophoresis, stain the gel with 0.5 $\mu\text{g/mL}$ ethidium bromide, and proceed from **step 5** of **Subheading 3.7.1**.

4. Notes

1. Accurate pH control is essential for the preparation of extracts with high repair and low nuclease activity. Unless otherwise stated, the pH values given in the protocol are for the indicated temperatures. It is important to keep in mind that the pH of Tris-buffered solutions changes significantly with temperature (it increases as the temperature decreases). Thus, a solution adjusted to pH 7.0 at room temperature will reach a pH of nearly 7.5 at 4°C. We found it very helpful to adjust the pH at the temperature under which each solution will eventually be used.
2. In the method described here, cells are radioactively labeled with ^{14}C -thymidine for quantitation. Quantitation of radioactively labeled DNA can be carried out

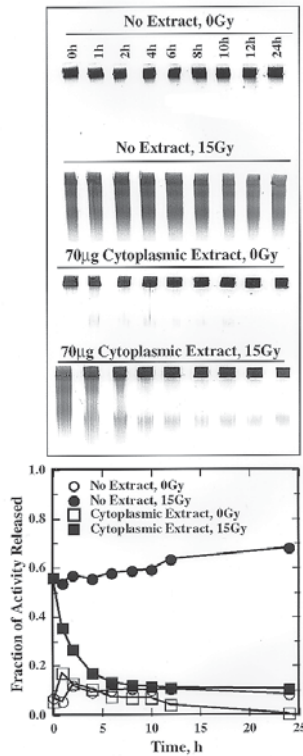


Fig. 2. Analysis of DSB rejoining in genomic DNA. The upper panel shows a series of four gels stained with ethidium bromide. The lower panel shows the results of their quantitation using a PhosphorImager. Open symbols represent nonirradiated samples, and closed symbols represent samples exposed to 15 Gy of X-rays just before assembling the repair reactions. A reduction in FAR indicates an increase in genomic DNA size as expected after DSB repair. Notice that the FAR is very low for samples not exposed to radiation, suggesting low nuclease activity. Notice also that in irradiated samples, the FAR decreases only when extract is present in the reaction.

either using a scintillation counter or a PhosphorImager. We prefer the latter instrument, since it is less labor-intensive. It is also possible to use fluorescence detection methods for quantitation (e.g., a FluorImager). If extreme sensitivity of detection is required, Southern blotting can also be considered (e.g., **10**). These alternative detection methods can easily be fitted into the described protocol. However, care should be exercised to ensure a linear response for the parameter measured within the range of interest.

3. Since usually a large number of reactions need to be assembled, we prepare a 10X stock of the reaction buffer by mixing stocks of the individual components. Subsequently, we setup reactions by adding the appropriate amount of 10X reac-

- tion buffer (5 μ L for a 50- μ L reaction), the desired amount of extract, and enough H_2O to reach a final volume of 50 μ L.
4. The preparation of a good extract depends strongly on the quality of the cells used. When cell growth is not optimal or when cells overgrow, high nuclease activity may severely compromise the use of the extract in repair reactions. To assure optimal growth, we routinely take the following measures:
 - a. Carefully test different batches of serum to find one with good growth characteristics. HeLa cells have a generation time of less than 20 h when grown as a monolayer and <24 h when grown in suspension, under optimal growth conditions.
 - b. Use cells grown in dishes to start the suspension cultures for extract preparation. This helps reduce cell clumping after extensive growth in suspension.
 - c. We follow cell growth daily, and collect cells for extract preparation when they reach a concentration of $4\text{--}6 \times 10^5$ cells/mL.
 - d. We measure cell-cycle distribution by flow cytometry. A high percentage of S-phase cells (~25% for HeLa cells) suggests that the cell culture is still in an active state of growth.
 5. The choice of A549 cells for substrate preparation is arbitrary and is guided by their property to produce cultures with a high percentage of G_0/G_1 cells when reaching the plateau phase of growth. Other cell lines can also be used if desired, but the protocol may require some modifications to accommodate the specific properties of these cells. When optimizing for the preparation of naked genomic DNA, the parameter to watch is degradation of nondamaged DNA during incubation under standard reaction conditions, but in the absence of extract; the FAR should be <10%. Relatively high FAR values under these conditions indicate the action of residual nucleases which should be removed by further lysis.
 6. A large number of agarose blocks with naked DNA can be prepared as a set and used, as needed, over a period of more than 1 yr. This gives a great degree of flexibility in the planning of this type of repair experiment. Extensive storage of nuclei embedded in agarose is not possible.

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Extrachromosomal Assay for DNA Double-Strand Break Repair

Feng Liang and Maria Jasin

1. Introduction

DNA double-strand break (DSB) repair in mammalian cells has been demonstrated to be complex, involving both homologous and nonhomologous processes. Although manipulation of chromosomal DSBs and analysis of their repair are possible (*1*; see Chapters 37–39), this is usually time-consuming, requiring the establishment and expansion of cell lines. To circumvent this we have refined an extrachromosomal assay to study both homologous and nonhomologous DSB repair processes in mammalian cells (*2,3*). The assay is not only useful to study general mechanisms of DSB repair in different mammalian culture systems, but can also be applied to the classification and characterization of molecular defects in repair-deficient mammalian cells (*3*).

The central feature of the assay is that it permits direct analysis of extrachromosomal substrates containing repaired DSBs after their transfection into, and subsequent recovery from, mammalian cells. The analysis may be performed statistically with bacterial transformation of the whole pool of recovered DNA, or in great detail by studying individual end products by restriction analysis and DNA sequencing. The three-plasmid assay is outlined in **Fig. 1**. Plasmid pBRtet is introduced uncleaved into mammalian cells, to serve as a transfection and transformation control. It confers tetracycline resistance (Tet^R) to bacteria when recovered from the transfected mammalian cells (*3*). Plasmids M5neo and M3neo are introduced as linear fragments into mammalian cells, and are used to measure extrachromosomal recombination and DNA end joining (*2,3*). Plasmid M5neo is cleaved with *Sph*I (M5neo/S) and plasmid M3neo is cleaved with *Aat*II and *Pst*I (M3neo/AP). The plasmid backbones of M5neo/S and M3neo/AP are each capable of being recircularized in mammalian cells

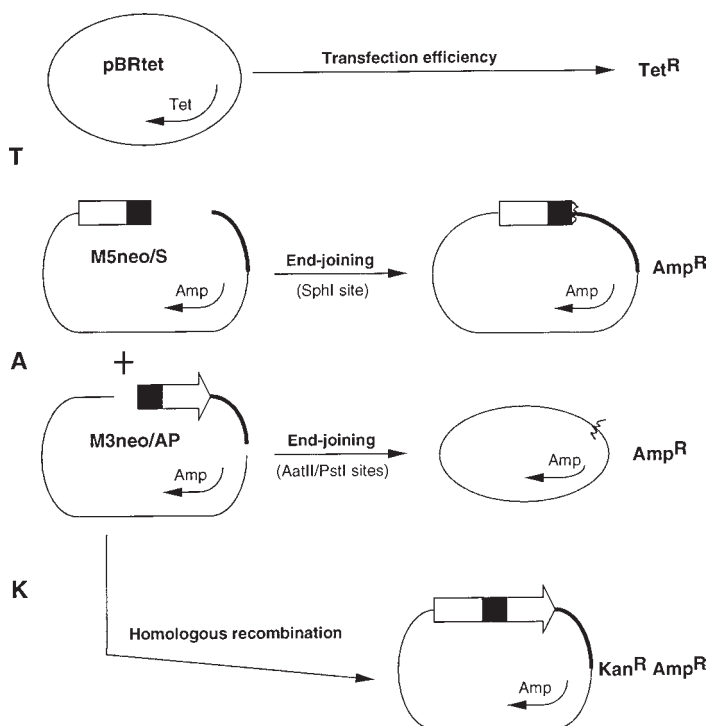


Fig. 1. The TAK assay for extrachromosomal DSB repair analysis. Plasmid substrates are transfected into mammalian cells, recovered after 4 h, and then transformed into bacteria. Plasmids confer Tet^R, Amp^R, and Kan^R as a measure of transfection efficiency and DNA stability, DNA end-joining, and homologous recombination, respectively. Plasmid pBRtet, which confers Tet^R, is introduced uncleaved and serves as the transfection control. Plasmids M5neo and M3neo are introduced cleaved with *SphI* (M5neo/S) and *AatII* and *PstI* (M3neo/AP), as indicated. Rejoining of plasmid ends confers Amp^R to bacteria. Recombination within the *neo* gene of M5neo/S and the homologous fragment from M3neo/AP additionally confers Kan^R to bacteria. (Since circular recombinants are a fraction of end joining products, they do not significantly contribute to the Amp^R pool of bacteria.) The downstream DSB in the plasmid sequences (thick line) can be healed by recombination (as shown) or end-joining (not shown). Open bar—*neo* gene; black shading—homology region of the *neo* gene.

to measure DNA end joining (2,3). The *SphI* 3'-overhangs of M5neo/S can be precisely or imprecisely rejoined, whereas the two different 3'-overhangs of M3neo/AP can only be imprecisely rejoined. When recovered from mammalian cells and reintroduced into bacteria, the recircularized plasmids confer ampicillin resistance (Amp^R), which serves as the measure of DNA end joining.

Individual end joining events can be studied and classified by analyzing plasmids from ampicillin-resistant transformants with restriction enzymes and DNA sequencing.

The M5neo and M3neo plasmids can also be used to measure DSB-promoted homologous recombination in mammalian cells (**Fig. 1**). M5neo contains the 3'-truncated Tn5 *neo* gene, and M3neo contains the 5'-truncated portion, with 352 bp of homology between them in the middle of the *neo* gene. The plasmid backbones are also homologous, although their recombination is not the focus of the assay. When the two DNAs recombine within the *neo* homology region (and have rejoined or recombined within the downstream plasmid sequences), a *neo*⁺ gene is recovered, and the recombined circular plasmid confers kanamycin resistance (Kan^R) to bacteria. Homologous recombination between two circular plasmids is inefficient in mammalian cells, but is greatly stimulated when a DSB is introduced next to the homology region. Thus, when M5neo is linearized with *Sph*I and M3neo is cleaved with *Aat*II and *Pst*I to release the homologous *neo* fragment (**Fig. 1**), recombination is readily detected (2). This three-plasmid assay is termed the "TAK" assay, since Tet^R measures transfection efficiency, Amp^R measures DNA end joining, and Kan^R measures homologous recombination.

The development of the TAK assay refines several extrachromosomal or in vitro assays developed previously (4,5). Compared to these assays, our system focuses more on the initial repair product. The plasmids and their derivatives do not contain a mammalian replication origin and, therefore, are not replicable in mammalian cells, which ensures that repair products will not be amplified before recovery. The transfected DNA is usually recovered within several hours after transfection to allow a more direct analysis of the repair process. The relatively short time period between transfection and recovery also makes it possible to compare cell lines with different division times. One other feature is that the Tn5 *neo* gene only expresses in bacteria, so it bypasses any potential transcriptional effects on DSB repair in mammalian cells.

Modified versions of the TAK assay can be applied to more specific questions in DSB repair (**Fig. 2**). For example, DNA end joining is suggested to involve complex activities, i.e., DNA end protection, end processing, ligation, and so forth. A genetic assay can be developed to focus more on a certain step. M3neo/AP is a good substrate for imprecise end joining, since the two overhangs are not compatible, and thus, modification of the ends is involved to recircularize the plasmid (**Fig. 2A**). On the other hand, *Pst*I-cleaved Mneo (Mneo/*Pst*I) can be repaired by both precise ligation of the two *Pst*I overhangs as well as imprecise end joining (**Fig. 2B**). In this design, the ratio of the two pools of repaired products (Kan^R vs Amp^R) is an important measure of the relative precision of end joining and can be altered in certain mammalian mutants

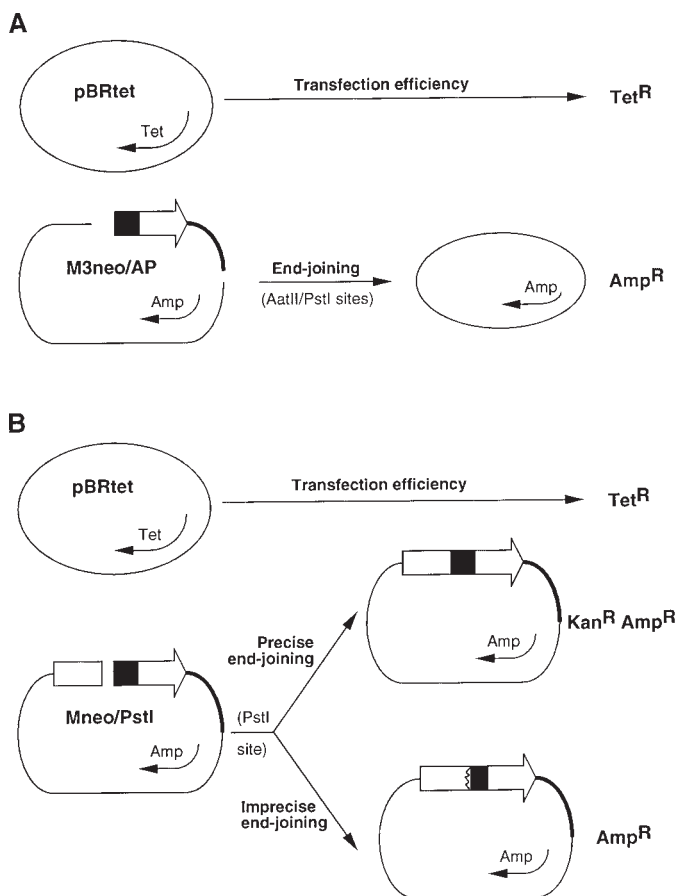


Fig. 2. Modified TAK assays. (A) An assay specifically for imprecise end-joining. Plasmid M3neo is introduced cleaved with *AatII* and *PstI* (M3neo/AP), as indicated. Rejoining of the incompatible sticky ends of the plasmid confers **Amp^R** to bacteria. Plasmid pBRtet, which confers **Tet^R**, is introduced uncleaved and serves as the transfection control. (B) An assay for precise ligation. Prior to transfection, Mneo is cleaved by *PstI*. Precise end joining gives rise to **Kan^R Amp^R** bacteria on transformation, whereas imprecise end-joining gives rise only to **Amp^R** bacteria. As before, plasmid pBRtet serves as the transfection control.

deficient in DSB repair (3). Different versions of the TAK assay may be useful to determine the defect in the end joining process in different mammalian cell mutants and shed light on the mechanism of repair. Although not discussed here, analysis of recovered DNA can be performed without passage through bacteria by Southern blotting to verify results obtained by bacterial transformation (2,3).

2. Materials

All solutions should be made according to the corresponding standard of molecular biology or tissue culture. The following list does not include some items commonly found in a laboratory.

2.1. Preparation of Extrachromosomal DNA Substrates

1. Plasmid Mneo: Plasmid Mneo was constructed by cloning the 1206 bp *HindIII/BamHI* Tn5 *neo* gene fragment from pSV2neoM7 into *HindIII/BamHI*-cleaved pUC19 (2). This plasmid contains an intact Tn5 *neo* gene in the pUC19 backbone and therefore confers both kanamycin and ampicillin resistance to bacteria.
2. Plasmid M5neo: M5neo was constructed by inserting the 885-bp *HindIII/SphI* Tn5 *neo* gene fragment of pSV2neoM7 into *HindIII/SphI*-digested pUC19 (2). This plasmid contains a 3'-truncated Tn5 *neo* gene in a pUC19 backbone and, therefore, only confers ampicillin resistance to bacteria.
3. Plasmid M3neo: Plasmid M3neo was generated by ligating the 673-bp *PstI/BamHI* *neo* gene fragment of pSV2neoM7 into *PstI/BamHI*-digested pUC19 (2). This plasmid contains a 5'-truncated Tn5 *neo* gene in a pUC19 backbone and, therefore, only confers ampicillin resistance to bacteria. The M5neo and M3neo plasmids have 352 bp of homology between the *PstI* and *SphI* sites of the *neo* gene.
4. Plasmid pBRtet: pBRtet was generated from pBR322 by deleting the 311-bp *ScaI/SspI* fragment of the ampicillin resistance gene (3). Therefore, it only confers tetracycline resistance to bacteria.
5. TE: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.
6. Restriction enzyme with corresponding 10X reaction buffer from supplier.
7. Buffered phenol.
8. Chloroform.
9. 3M Sodium acetate, pH 5.2.
10. Ethanol, 100%.
11. Ethanol, 80%.
12. TBE buffer: 90 mM Tris-borate, 2 mM EDTA.
13. Agarose.

2.2. Transfection of Mammalian Cells and Recovery of Extrachromosomal DNA

Cell lines are cultured in 150-cm² tissue-culture flasks and transfected by electroporation. Flasks are preferred for easy manipulation of the large amount of cells needed for efficient recovery of transfected extrachromosomal DNA.

1. Tissue-culture medium.
2. Serum.
3. Penicillin–streptomycin solution: 100X stock available from supplier (Gibco BRL, Gaithersburg, MD).

4. Phosphate-buffered saline (PBS), pH 7.4.
5. Trypsin-EDTA solution: 10X stock available from supplier (Gibco BRL).
6. Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA).
7. Bio-Rad Gene Pulser cuvet: 0.4 cm.
8. Tris/NaCl/EDTA buffer: 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA.
9. Phenol/chloroform/isoamyl alcohol mixture: 25:24:1.
10. 4 M Ammonium acetate.
11. Ethanol, 80%.
12. Ethanol 100%.

2.3. Preparation of *E. coli* DH10B for Electrotransformation (see Note 1)

1. *E. coli* DH10B, genotype F⁻ *mcrA* Δ (*mrr-hsdRMS-mcrBC*) Δ 80d*lacZ* Δ M15 Δ *lacX74 deoR recA1 end A1 araD139 Δ (*ara,leu*)7697 *galU galK* λ^- *rpsL nupG* (Life Technologies).*
2. LB medium: 1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl, pH 7.0.
3. Glycerol, 10%.

2.4. Analysis of Recovered Extrachromosomal DNA by Bacterial Transformation

1. *E. coli* DH10B cells for electrotransformation.
2. SOC medium: 2% Bacto-tryptone, 0.5% Bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose, pH 7.0.
3. Bio-Rad Gene Pulser.
4. Bio-Rad Gene Pulser cuvette: 0.1-cm.
5. Tetracycline-containing LB agar plates.
6. Ampicillin-containing LB agar plates.
7. Kanamycin-containing LB agar plates.
8. LB medium: 1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl, pH 7.0.
9. 3 M Sodium acetate, pH 5.2.
10. Ethanol, 80%.
11. Ethanol, 100%.
12. TE: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.
13. Restriction enzyme with corresponding 10X reaction buffer.
14. TBE buffer: 90 mM Tris-borate, 2 mM EDTA.
15. Agarose.
16. DNA sequencing materials.

3. Methods

3.1. Preparation of Extrachromosomal DNA Substrates

DNA manipulations are performed according to standard procedures (6). The following protocol is scaled to one sample, which needs to be multiplied if several mammalian cell lines are going to be compared or if a time-course is

involved. One hundred fifty micrograms of each of the following plasmid substrates are suggested for individual transfection (*see Note 2*).

1. Supercoiled pBRtet.
2. *Sph*I-cleaved M5neo.
3. *Pst*I/*Aat*II-cleaved M3neo.

3.2. Transfection of Mammalian Cells and Recovery of Extrachromosomal DNA

1. Culture mammalian cell lines in 150-cm² tissue-culture flasks. 10⁸ cells are suggested for each transfection.
2. Harvest cells at subconfluence with trypsin-EDTA.
3. Wash the cells with PBS.
4. Resuspend the cells in PBS at 1.6 × 10⁸ cells/mL. Mix 10⁸ cells (about 0.6 mL) with 150 µg of each plasmid (pBRtet, M5neo/S, and M3neo/AP) in a final volume of 0.8 mL (*see Note 3*).
5. Place the DNA/cell mixture in a 0.4-cm Gene Pulser cuvette, and immediately electroporate using a Bio-Rad Gene Pulser with a setting of 250 V and 960 µF.
6. Wash the transfected cells twice in medium after electroporation to remove untransfected DNA.
7. Incubate the transfected cells in tissue-culture medium at 37°C.
8. Harvest the cells with trypsin-EDTA after 4 h or another desired time of incubation (*see Note 4*).
9. Wash the cells three times in PBS to remove remaining untransfected DNA.
10. Spin down the cells at 3000 rpm (approx 720g) in a microcentrifuge at room temperature.
11. Resuspend the cell pellet in 100 µL of Tris/NaCl/EDTA buffer (*see Note 5*).
12. Add 100 µL phenol/chloroform/isoamyl alcohol mixture, and vortex for 15 s.
13. Spin at 14,000 rpm (15,800g), and then transfer the supernatant to a fresh Eppendorf tube avoiding the interphase as much as possible.
14. Add an equal volume of 4 M ammonium acetate and 5 vol of prechilled 100% ethanol, and allow the DNA to precipitate at -20°C overnight.
15. Spin down the precipitate at 4°C.
16. Wash the DNA pellet with 80% ethanol (*see Note 6*).
17. Dry the pellet completely.
18. Resuspend the pellet in 50 µL of H₂O. The DNA sample is ready for analysis.

3.3. Preparation of E. coli DH10B for Electrotransformation (see Note 1)

Bacteria are grown to early- to mid-log phase and then washed extensively with 10% glycerol to lower the ionic strength of the cell suspension. The salt-free cells are resuspended in 10% glycerol at a high concentration (1–3 × 10¹⁰ cells/mL) and stored at -80°C to be used for electrotransformation for up to half a year without much decrease in transformation efficiency.

1. Grow *E. coli* DH10B in 1 L of LB medium at 37°C until the OD₆₀₀ reading reaches 0.4–0.6, and then chill the bacteria on ice for 15 min.
2. Recover the cells by centrifuging at 2000g for 15 min at 4°C.
3. Wash the pellet in 1 L of 10% glycerol, then recover the bacteria by centrifuging at 2000g for 15 min at 4°C.
4. Wash the cells in 250 mL of 10% glycerol, and then centrifuge at 2000g for 15 min at 4°C.
5. Wash the cells in 80 mL of 10% glycerol, and then centrifuge at 2000g for 15 min at 4°C.
6. Wash the cells in 10 mL of 10% glycerol, and then centrifuge at 2000g for 15 min at 4°C.
7. Resuspend the pellet in 1 mL of 10% glycerol, freeze in aliquots of 100 µL on dry ice, and store them at –80°C.
8. Test the transformation efficiency of the cells. The overall transformation efficiency should be over 10⁹ colonies/µg with 20 µL of cells and 0.5 ng of pBRtet.

3.4. Analysis of Recovered Extrachromosomal DNA by Bacterial Transformation (see Note 7)

1. Gently thaw the bacterial cells prepared in **Subheading 3.3.**, and immediately place the tube on ice.
2. Set the Bio-Rad gene pulser apparatus at 1.7 kV and 25 µF, and set the Pulse Controller to 200 Ω.
3. Mix 20 µL of bacteria with 0.5 µL of the DNA sample obtained in **Subheading 3.2.**, and pulse in a prechilled 0.1-cm cuvette at the above settings (*see Note 8*).
4. Immediately add 1 mL of 37°C prewarmed SOC medium to the cuvet, and resuspend the cells (*see Note 9*).
5. Transfer the cell suspension to a polypropylene tube, and shake the transformed bacteria at 225 rpm for 1 h at 37°C.
6. Plate the appropriate amount of bacteria onto two LB agar plates containing tetracycline. Repeat for two LB agar plates with ampicillin and two with kanamycin (*see Note 10*).
7. Leave the plates at room temperature until the liquid has been absorbed.
8. Invert the plates, and incubate at 37°C overnight until colonies are visible, but not overgrown.
9. Count the colonies on all six plates.
10. Grow up individual ampicillin- or kanamycin-resistant transformants in selective liquid medium, and prepare plasmid DNA according to standard procedures (**6**; *see Note 11*).
11. Analyze the plasmid by restriction digests and DNA sequencing (*see Note 12*).

4. Notes

1. Commercially prepared cells are also available from Life Technologies.
2. Complete digestion of extrachromosomal substrates is critical for the TAK assay. Contamination of circular plasmids in linearized substrate DNA preparations will

result in a high background in the bacterial transformation assay, interfering with the ability to measure mammalian DSB repair. Therefore, it is recommended to check the digestion on an agarose gel with overloaded samples or by bacterial transformation, since linearized plasmids transform bacteria poorly (three to five orders of magnitude lower than its circular counterpart; **ref. 2**). Purification of the linearized product will be needed for some plasmids, e.g., *Pst*I-cleaved Mneo.

3. Efficient recovery of extrachromosomal DNA in transfected mammalian cells is largely dependent on the amount of cells electroporated and the amount of input DNA. In general, the more of both, the better the recovery. However, both factors are relatively flexible; one can be compensated for by the other. In this protocol, high concentrations of cells and DNA substrates are recommended. At the high concentrations, the mixture will be very viscous. However, it is necessary to mix the cells and DNA gently to avoid air bubbles, which interfere with electroporation.
4. The time to harvest the cells is critical, although the best time window may vary among cell lines. After transfection, the overall amount of recovered extrachromosomal DNA decreases owing to DNA degradation (**3**). However, the repaired products appear with a transient peak at a few hours after transfection in cells that have been tested (**2,3**). It is recommended at the start of these experiments that a time-course be performed in the transfected cells (up to 24 h or more of incubation) to determine the best time(s) for a specific cell line. Overall, we prefer a relatively short time of incubation of the transfected cells (4 h) if it is permissible.
5. In general, it is suggested that the volume of Tris/NaCl/EDTA buffer be at least equivalent to the volume of the cell pellet. Too small a volume of Tris/NaCl/EDTA buffer will result in a small aqueous phase compared to a much larger interphase after extraction with the phenol/chloroform/isoamyl alcohol mixture.
6. The amount of precipitate is usually proportional to that of the cell pellet and may be difficult to see. The precipitate is composed mostly of RNA, which has little effect on bacterial transformation. Washing the precipitates with 80% ethanol is critical, given that DNA samples with too much salt will be conductive and cause arcing during bacterial electroporation.
7. The recovered extrachromosomal DNA in this procedure is usually in the nanogram range, which is more than enough for several rounds of bacterial transformation and Southern analysis, if desired. Southern analysis may be performed using 1–2 μ L DNA (2–4%) according to standard procedures (**6**). With proper restriction digestion, Southern analysis can reveal end products of both recombinational repair and end joining, and even some bimolecular intermediate products in which only one set of ends has been repaired, providing the most direct analysis of DNA DSB repair. Based on information provided by Southern analysis, PCR may be performed to amplify and analyze repaired products. Bacterial transformation, although a more indirect approach, provides more accurate sta-

tistical information on repaired products than does Southern analysis. The end products of DNA repair can be conveniently studied in detail in the latter experimental procedure. Therefore, it is strongly recommended to use both Southern analysis and bacterial transformation to analyze recovered DNA even though the protocol emphasizes the latter. Throughout the analysis, proper controls should be included, e.g., untransfected DNA substrates.

8. Bacterial electroporation is most efficient when it is carried out at low temperature (0–4°C). Therefore, it is suggested to mix the DNA with the cells on ice, and transfer the mixture to an ice-cold electroporation cuvette just before electroporation.
9. The rapid addition of prewarmed SOC medium to the bacterial cells after the pulse is critical for maximizing the recovery of transformants.
10. It is suggested to use duplicate samples for electroporation, and to plate duplicate plates for the individual “T,” “A,” and “K” signals in this statistical assay. The transformants on individual plates should be reasonably dense, but not hinder the counting and picking of individual colonies. Several hundred transformants are suggested for each 100-mm plate. The amount of bacterial cells to be plated with each antibiotic may need to be pretested, but a reasonable starting amount would be 450 μ L on kanamycin, 50 μ L on ampicillin, and 1 μ L on tetracycline. These amounts can be altered based on the transformation efficiency of the bacteria being used.
11. Analysis of individual ampicillin- or kanamycin-resistant transformants is aimed at revealing the nature of specific end products of repair in mammalian cells. Linear DNA can also be repaired in bacteria, although with a very low efficiency. A significant differential of transformation efficiency should always be observed between DNA that has been transfected in mammalian cells first and then transformed into bacteria and DNA transformed directly into bacteria (2). With this differential, repair events can be assumed to have occurred in mammalian cells.
12. Restriction analysis is convenient for studying a large number of transformants. It can confirm homologous recombination and reveal useful information about end joining, such as reCleavability of the initial cleavage site and the size of the repaired product. With this information, limited DNA sequencing can be performed to reveal the details of end joining products. Among the common events of end joining are deletions and insertions, and, more rarely, base substitutions near the initial DSB.

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Use of Gene Targeting to Study Recombination in Mammalian DNA Repair Mutants

Rodney S. Nairn and Gerald M. Adair

1. Introduction

Gene targeting, defined as homologous recombination or genetic exchange between an introduced DNA sequence and its endogenous chromosomal locus, or “target,” is a powerful approach for genetic manipulation. Gene-targeting strategies for both yeast (*1*) and mammalian cells (*2–4*) have been described that allow correction, disruption, deletion, replacement, or site-directed modification of virtually any gene or chromosomal locus for which a cloned sequence is available. The majority of mammalian gene-targeting studies have been directed toward disruption (“knockout”) of a selected target gene locus in mouse embryo stem (ES) cells (*4,5*), with the primary objective of obtaining the desired mutant mouse as quickly as possible. Relatively few studies have examined targeted recombination in cell types other than mouse ES cells. This chapter describes methods and use of targeted recombination as an approach to study mechanisms of recombination in cultured mammalian cells, and, in particular, the use of gene-targeting approaches to generate and analyze DNA repair-deficient knockout mutants to reveal interactions between DNA repair and recombinational pathways in mammalian cells.

In budding yeast, it is well established that a number of DNA repair genes, including at least two nucleotide excision repair (NER) genes, *RAD1* and *RAD10*, encode proteins with dual roles in DNA repair and genetic recombination (*6–9*). In our research, we have focused on the interaction between NER and recombination pathways in mammalian cells, by investigating genetic recombination in excision repair cross-complementing, group 1 (*ERCC1*) mutants isolated from Chinese hamster ovary (CHO) cells, using a variety of approaches (*10–13*). Since the *Ercc1* polypeptide exhibits extensive homology

with the yeast *RAD10*-encoded protein, these studies were designed to reveal similar recombination-deficient phenotypes in CHO cell *ERCC1* mutants and yeast *RAD10* mutants. Although conventional *ERCC1* mutants isolated after chemical mutagenesis have been very useful in these studies, we recently focused on construction of *ERCC1* knockout mutants to address the general question of the role of NER proteins in genetic recombination in mammalian cells (*12a,14*; unpublished results). The advantages of using knockout mutants generated in well-characterized cell lines for such studies include:

1. Many types of molecular studies are best conducted in vitro in cultured cells, rather than in whole animals.
2. The risk of introducing secondary, cryptic mutations that may influence experimental outcomes is minimal compared to conventional mutagenesis with chemical mutagens.
3. Pairs of somatic cell lines, one generated by gene disruption and its parental line, should be essentially isogenic and differ only with respect to the disrupted target gene.

Particular advantages of CHO cells for such studies are:

1. CHO cells exhibit balanced ploidy, are easily grown and maintained, and have a high cloning efficiency.
2. CHO cells are widely used in somatic cell genetics (*15*), and a large data base exists in the literature describing mutagenic and cytotoxic responses of CHO cells to a variety of physical and chemical agents (*16*).
3. A number of gene loci are hemizygous in CHO cell lines, including several DNA repair genes (*14,17,18*). Hemizygous gene loci greatly simplify both the generation of targeted gene knockouts and the molecular analysis of gene alterations generated from gene targeting.

The following subsections describe the underlying principles of targeted gene disruption, applied in particular to CHO cells, and the utility of the hemizygous CHO *APRT* gene as a model target locus for gene-targeting studies designed to probe the mechanisms of mammalian recombination. As an example of the use of gene targeting to create knockout mutants, targeted gene disruption of the *ERCC1* DNA repair gene in CHO cells is described; however, other DNA repair genes from both the NER and double-strand break repair (DSBR) pathways should be suitable candidates for knockout in CHO cells by gene-targeting approaches, and the approaches described for knockout of *ERCC1* should be generally applicable to other cloned DNA repair genes.

1.1. Targeted Knockout of a DNA Repair Gene Locus in Cultured Mammalian Cells

It has been shown that isogenic sequence (i.e., DNA isolated from the particular cell line to be used for gene targeting) is necessary for efficient targeted

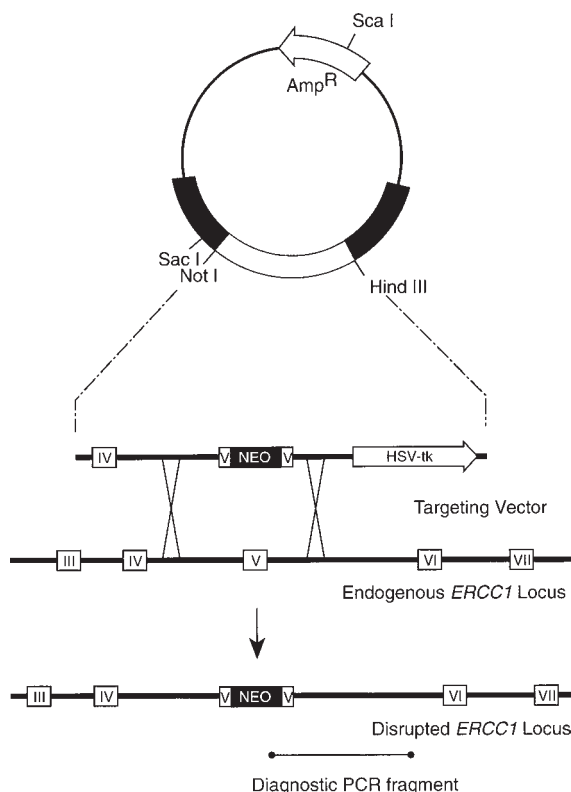


Fig. 1. Targeted gene knockout strategy for gene disruption of the *ERCC1* locus in CHO cells.

recombination in mammalian cells (19,20). Therefore, for targeted gene disruption of the CHO *ERCC1* gene, a human cDNA *ERCC1* probe (21) was used to screen a CHO cell genomic library constructed in Lambda FIXII (Stratagene, La Jolla, CA), resulting in recovery of the CHO *ERCC1* gene. A fragment containing exons 4 and 5 was subcloned and used to construct a targeting vector (see Fig. 1); this vector was used for targeted gene knockout in the CHO K-1 cell line as described in Subheading 3. (14), and also has been used for *ERCC1* gene knockout in several other CHO cell lines derived from the CHO-AT3-2 cell line (12a).

Several parameters of gene targeting should be taken into consideration in designing a knockout vector. A study of gene targeting at the *HPRT* locus in mouse ES cells (20) showed that targeting vector configuration (i.e., insertion vector configuration, having a double-strand break (DSB) within target gene sequence homology in plasmid DNA vs replacement vector configuration, with

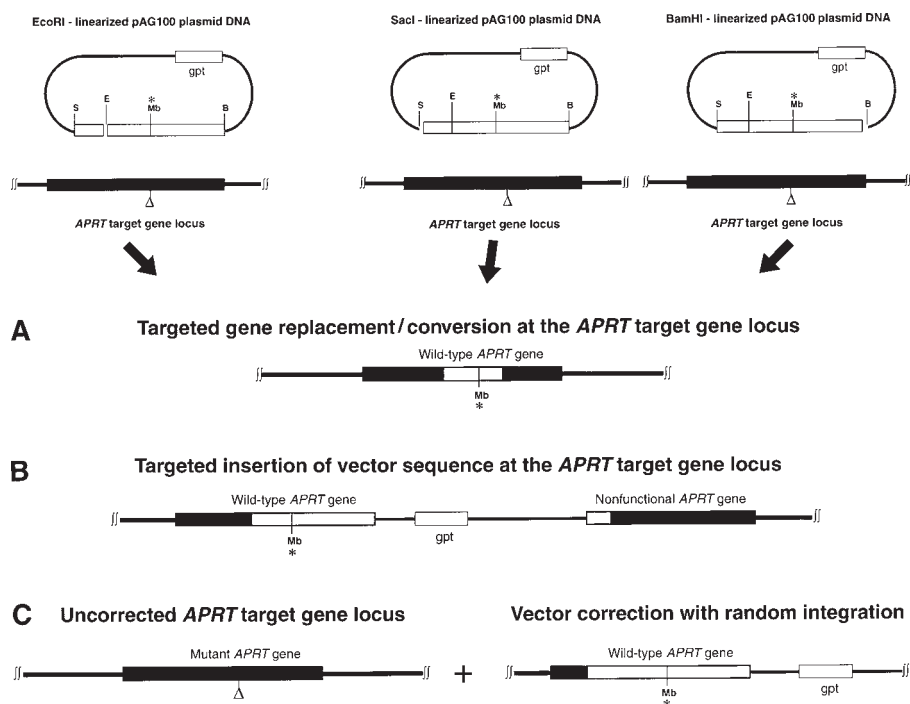


Fig. 2. Targeted recombination at the CHO-ATS49tg *APRT* locus.

a DSB in the targeting vector outside target gene sequence homology in plasmid DNA) is less important for efficient targeted gene disruption than the fact that the homologous DNA sequence in the targeting vector is isogenic. In CHO cells, we have conducted experiments with both insertion-type and replacement-type *APRT* targeting vectors that support this finding, showing that the overall efficiency of gene targeting is essentially the same for both insertion-type and replacement-type vectors (12,21a). However, the distribution of gene-targeting events can be dramatically affected by targeting vector configuration. Use of a replacement-type targeting vector greatly reduces targeted insertion recombinants compared to gene-replacement/conversion recombinants (12,21a; see Fig. 2 for generalized structures of targeted insertion and gene-replacement recombinants). Therefore, insertion-type targeting vectors are most efficient for two-step gene replacement or modification strategies (21a–24), which require targeted insertion in the first step, whereas replacement-type vectors may be preferable for gene disruption or knockout, gene-targeting approaches.

Another factor critical for efficient gene targeting is the length of target gene sequence homology in the targeting vector. A minimum length of sequence

homology is necessary to flank each side of the positive selection marker (e.g. *NEO*) in a replacement-type targeting vector (see **Fig. 1**). Targeting efficiency decreases rapidly with decreasing length of sequence homology below about 500 bp (25), suggesting that a minimum of 500–600 bp of homologous sequence flanking each side of *NEO* (or whatever selectable marker is used) should be designed into the knockout vector; typically, several kilobases of homologous sequence flank each side of the selection marker (26). Negative selection markers, such as *HSV-tk*, are used in positive–negative selection protocols to enrich the population of transformed cells for targeted homologous recombinants (27). Selection against *HSV-tk* using gancyclovir or FIAU applied to the transfected cell population kills nonhomologous recombinants in which the targeting vector has randomly integrated into genomic DNA; gene-replacement events that have lost the *HSV-tk* marker during recombination are thus enriched in a positive–negative selection protocol using *NEO* and gancyclovir or FIAU (**Fig. 1**). Although very large enrichment ratios using positive–negative selection (up to three orders of magnitude) have been reported (27), the degree of enrichment attainable depends on the target locus, the cell type, and the targeting vector; in practice, usually between one and two orders of magnitude of enrichment is reasonable to expect.

1.2. Use of APRT Gene-Targeting Assays to Study Recombination Deficiencies in Mammalian Cells

We have developed practical mammalian gene targeting assay systems utilizing hemizygous, CHO *adenine phosphoribosyltransferase* (*APRT*) deletion mutants, such as CHO-ATS49tg, for studying homologous recombination in cultured mammalian cells (12,12a,13,21a,22,25,28,29). These CHO-targeted recombination/*APRT* gene correction assays employ either insertion-type or replacement-type targeting vector configurations and allow direct selection of *APRT*⁺ recombinants. Our own work and work from other laboratories have demonstrated the utility of the hemizygous CHO *APRT* gene as a model target locus for gene-targeting studies designed to probe the mechanisms of mammalian recombination (12,21a,22,25,28–34). Use of a hemizygous, nonrevertible, deletion-inactivated *APRT* gene as a target locus ensures that *APRT*⁺ cells can arise only by targeted homologous recombination at the endogenous *APRT* gene locus. The small size of the CHO *APRT* gene (which spans only ~2.2 kb from promoter to polyadenylation signal), absence of *APRT* pseudogenes, and hemizygosity for the target gene locus, all greatly facilitate molecular analysis of *APRT*⁺ recombinants.

Three distinct classes of recombinants can be recovered from such *APRT*-targeting experiments, reflecting at least three different types of targeted homologous recombination events:

1. Target gene conversions.
2. Targeted insertions at the chromosomal *APRT* gene locus.
3. Targeting vector corrections, involving target gene-templated extension and correction of the targeting vector *APRT* sequence (28).

These three recombinant classes are readily distinguishable on the basis of their distinctive and diagnostic restriction fragment patterns. Target gene convertants (**Fig. 2A**) show simple correction of the ATS49tg target gene deletion, with precise replacement of the three deleted bases and restoration of the exon5 *Mbo*II restriction site. Targeted insertions of the pAG100 targeting vector, produced by single-crossover, reciprocal exchange events (**Fig. 2B**), result in a partial duplication of *APRT* gene sequences at the ATS49tg target gene locus, generating a corrected wild-type copy of the *APRT* gene at one end (upstream) of the inserted pAG100 vector sequence, and a 5'-truncated *APRT* gene sequence at the other end (downstream). The downstream 5'-truncated *APRT* gene sequence can show either correction or retention of the 3-bp exon 5 *Mbo*II site deletion. The third class of recombinants (**Fig. 2C**), generated by target gene-templated extension and correction of the pAG100 targeting vector *APRT* sequence, contain both an uncorrected ATS49tg *APRT* gene at the original targeted chromosomal locus, and a second, wild-type recombinant *APRT* gene, which has been integrated elsewhere in the genome. These recombinants appear to arise by one-sided strand invasion by the *APRT* targeting sequence into the target gene duplex, followed by target gene-templated extension of the 3' hydroxy end of the invading strand to restore the promoter and 5' *APRT* region missing from the 5'-truncated targeting vector *APRT* sequence. This produces a corrected (wild-type) targeting vector *APRT* gene, which is then randomly integrated into the genome (21a,28).

Comparisons of gene-targeting frequencies and recombinant class distributions for DNA repair-proficient CHO-ATS49tg cells, and either conventionally derived CHO DNA repair-deficient mutants or isogenic, repair gene knockout cell lines, can provide valuable insights into the possible roles of specific mammalian repair gene products in various recombinational pathways. Although we have carried out *APRT*-targeting experiments using several conventionally derived *ERCC1* or *ERCC2* mutants, NER-deficient CHO cells lines (12,13, and unpublished data), it is quite difficult to obtain matched, repair-proficient and deficient cell lines that contain the same *APRT* target gene deletion. The advantages of using isogenic repair gene knockout cell lines for such studies are compelling. *APRT*-targeting experiments employing NER-deficient *ERCC1* mutant (U9S50tg) and repair-proficient (ATS49tg) cell lines that contain identical 3-bp *APRT* target gene deletions have revealed differences in the class distributions of targeted recombinants obtained, with the *ERCC1* mutant showing specific decreases in the relative frequencies of certain types of tar-

geted recombination events (**13**, and unpublished data). These experiments are being repeated using a CHO-ATS49tg-derived *ERCC1* knockout cell line. Our preliminary results, and results of direct-repeat recombination assays in CHO cells, suggest that the *ERCC1* NER gene may indeed play a role in the recombinational pathways of mammalian cells similar to that of the *RAD10* gene in yeast (**12a**, and unpublished data).

2. Materials

2.1. Knockout and APRT Gene Targeting

1. α MED-10% FCS medium: α -modified Minimal Essential Medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal calf serum (FCS), penicillin (50 U/mL), and streptomycin (50 μ g/mL). Penicillin and streptomycin (tissue culture grade) are available from Gibco BRL (Gaithersburg, MD) as well as other suppliers. A formulation of this medium using dialyzed FCS (i.e., α MED supplemented with 10% FCS that has been dialyzed against four changes of HBSS and filter-sterilized) is referred to as α MED-10% DFCS. Serum-free α MED is referred to as SFMED.
2. Puck's Solution A (solution A): Physiological saline (0.8% NaCl) in 5 mM KCl, 4.2 mM NaHCO₃, containing 0.1% dextrose. A 10X stock solution is prepared by dissolving 80 g NaCl, 4 g KCl, 10 g dextrose, and 3.5 g NaHCO₃ in 1 L of H₂O; a 1X working solution is used after 1:10 dilution with H₂O and filter sterilization. Store both 10X and 1X solutions at 4°C.
3. Hank's Balanced Salt Solution (HBSS): Physiological saline (0.8% NaCl) in 5.4 mM KCl, 0.33 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 4.2 mM NaHCO₃, 0.5 mM MgCl₂, 1 mM CaCl₂, containing 0.1% dextrose. A 1X solution is prepared by dissolving 40 g NaCl, 2 g KCl, 0.45 g Na₂HPO₄ · 7H₂O, 0.3 g KH₂PO₄, 1.75 g NaHCO₃, 0.5 g MgCl₂ · 6H₂O, 0.7 g CaCl₂ · 2H₂O, and 5 g dextrose in 5 L of H₂O. Filter-sterilize and store at 4°C.
4. Phosphate-buffered saline (PBS): Physiological saline (0.8% NaCl) in 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.5 mM KCl. A 10X solution is prepared by dissolving 12 g KCl, 12 g KH₂PO₄, 480 g NaCl, and 129.6 g Na₂HPO₄ · 7 H₂O in 6 L of H₂O. A 1X working solution is prepared by autoclaving a 1:10 dilution (in H₂O). Store at 4°C.
5. Trypsin-EDTA: 0.25% Trypsin, 25 mM Tris-HCl, 0.5 mM EDTA in solution A. Dissolve 3 g of Trizma base (Sigma) in 460 mL of solution A and add 20 mL of 1 N HCl; dissolve 0.2 g Na₂EDTA · 2 H₂O (Sigma, St. Louis, MO) in 20 mL water; dissolve 2.5 g trypsin (Gibco BRL) in 450 mL solution A, while stirring. Stir all three solutions individually until homogeneous, mix the Trizma and EDTA solutions together to dissolve, then add the trypsin suspension, and adjust to pH 7.4 if necessary. Adjust the final volume to 1 L with H₂O, filter-sterilize, and dispense in 10-mL aliquots. Store frozen (do not refreeze).
6. Tris-EDTA buffer (TE): 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. For a 10X stock solution, dissolve 12.1 g Trizma base (Sigma) and 3.7 g Na₂EDTA · 2H₂O in 950 mL of H₂O; adjust to pH 8.0 with HCl, and bring the final volume to 1 L; store at 4°C. For a 1X working solution, dilute 1:10 with H₂O and filter-sterilize.

7. 10% Sodium dodecyl sulfate (SDS): Dissolve 100 g of SDS in 1 L (final volume) of hot water with gentle stirring; autoclave and store at room temperature.
8. 5 M Lithium acetate: Dissolve 51 g $\text{Li}(\text{C}_2\text{H}_3\text{O}_2) \cdot 2\text{H}_2\text{O}$ in 100 mL H_2O final volume; autoclave and store at 4°C.
9. Equilibrated phenol: Melt crystalline, H_2O -saturated redistilled phenol (stored frozen) at 37°C in a water bath. Add one-half volume of 0.5 M Tris-HCl, pH 7.5, and stir at room temperature for 30–45 min. Separate and retain the lower, organic phase. Add 1 vol 0.1 M Tris-HCl, pH 7.5, shake in separatory funnel, separate, and retain the lower phase. Repeat once. Recover the equilibrated phenol, and store under a shallow layer of 0.1 M Tris-HCl, pH 7.5, in a brown glass bottle, for up to 3 wk at 4°C.
10. Crystal violet staining solution: A 10X stock solution is prepared by dissolving 20 g of crystal violet (Sigma) in 400 mL of 90% ethanol. A 1X working solution for staining is prepared by 1:10 dilution with 90% ethanol. Store at room temperature.
11. Restriction endonucleases: Available from Boehringer-Mannheim (Indianapolis, IN), Gibco BRL, Promega (Madison, WI), New England Biolabs (NEB, Beverly, MA), and Stratagene, and used according to the suppliers' directions.
12. Southern blotting reagents: QuikHyb Hybridization Solution (Stratagene) is used for Southern hybridizations, using double-stranded DNA probes labeled with α - ^{32}P -dCTP by nick translation or random primer labeling.

2.2. Knockout Gene Targeting in CHO Cells

1. Cell line: CHO-ATS49tg (*see Note 1*).
2. *ERCC1* targeting vector: pSL1 (*see Note 2*).
3. High-ionic-strength electroporation buffer (HEP buffer): 20 mM *N*-2-hydroxyethylpiperazine-*N'*-[2-ethanesulfonic acid] (HEPES), 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 , 6 mM dextrose, pH 7.05 (35). Just prior to electroporation, prepare a fresh solution by dissolving 238.5 mg HEPES, 400 mg NaCl, 54 mg dextrose, 18.5 mg KCl, and 9.5 mg $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ in 40 mL H_2O ; adjust the pH to 7.05 with 1 N NaOH. Adjust the final volume to 50 mL prior to filter sterilization.
4. G418/FIAU selection medium: 400 $\mu\text{g}/\text{mL}$ G418, 0.2 μM 1-[2-deoxy-2-fluoro- β -D-arabinofuranosyl-5-iodo]-uracil (FIAU) in α MEM–10% DFCS. Adjust the G418 (Geneticin, Gibco BRL) to 100% activity by weight, and dissolve directly in SFMEM to 20 active mg/mL (50X), then neutralize (to color) with 1 N NaOH, filter-sterilize, and store at 4°C. Prepare FIAU as a 0.2-mM stock by dissolving 4.6 mg of FIAU in 0.5 mL of fresh 1 N NaOH, and then bring the volume to 60 mL with PBS; after filter-sterilizing, aliquot the FIAU stock (1000X) and store frozen. Prepare selection medium containing G418 alone, or both G418 and FIAU, by appropriate dilutions directly into α MEM–10% DFCS (*see Note 3*). FIAU was the generous gift of Bristol-Myers Squibb Pharmaceutical Research Institute (Wallingford, CT).

5. DNA lysis solution: 1% proteinase K. Dissolve proteinase K lyophilized powder (Boehringer Mannheim) at 10 mg/mL in H₂O; stir or shake gently to avoid foaming. Prepare fresh just before use.
6. PCR primers: Forward Neo 1: 5'-CCGCTTTTCTGGATTCATCGAC-3', and Forward Neo 2: 5'-GCTTCCTCGTGCTTTACGGTATC-3' are nested in the pMC1neopA cassette (36); Reverse ERCC1: 5'-TGCCACCCCTGACCACATATACAC-3' was designed in a region of *ERCC1* not represented in the targeting vector, pSL1 (see Note 4). We use commercially prepared primers from Operon (Houston, TX) or Genosys (Alameda, CA).
7. PCR reagents: Reagents for PCR, including *Taq* DNA polymerase, were commercially purchased as a kit from Perkin-Elmer (GeneAmp PCR Reagent Kit, N801-0043). *Taq* Extender and *Taq*Start antibody were purchased from Stratagene and Clontech (Palo Alto, CA), respectively.

2.3. *APRT* Gene Targeting in CHO Cells

1. Cell line: CHO-ATS49tg (see Note 5).
2. *APRT* targeting vector: pAG100 (see Note 6).
3. Low-ionic strength electroporation buffer (LEP buffer): 272 mM sucrose, 1 mM MgCl₂, 7 mM sodium phosphate, pH 7.1 (Bio-Rad Gene Pulser Instruction Manual, Hercules, CA). Just prior to electroporation, prepare fresh electroporation buffer by dissolving 2.33 g of sucrose and 47 mg Na₂HPO₄ · 7H₂O in 20 mL of H₂O; add 25 µL of 1 M MgCl₂, adjust the pH to 7.1, and bring the final volume to 25 µL with H₂O prior to filter sterilization.
4. ALASA selection medium (ALASA): 25 µM alanosine, 50 µM azaserine, 100 mM adenine in αMEM–10% FCS (see Note 7). Prepare alanosine as a 12.5 µM (500X) stock by dissolving 93 mg alanosine in 1 mL of 1 N (fresh) NaOH; bring to 50 mL with Solution A, filter-sterilize, and store frozen in 2.5 mL aliquots (may be refrozen). Alanosine (NSC-529469) can be obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute. Prepare azaserine (Sigma) as a 25-mM (500X) stock by dissolving 216.5 mg 8-azaserine in 1 mL of 1 N (fresh) NaOH; bring to 50 mL with solution A, filter-sterilize, aliquot, and store frozen (may be refrozen). Prepare adenine (Sigma) as a 5 mM (50X) stock by dissolving 42.9 mg of adenine · HCl in 50 mL solution A. After filter-sterilizing, store at room temperature.
5. HAT selection medium (HAT): 100 µM hypoxanthine, 2 µM amethopterin, 50 µM thymidine in αMEM–10% FCS (see Note 8). Prepare hypoxanthine as a 50X stock by dissolving 68 mg hypoxanthine (Sigma) in 80 mL of H₂O; add 1 mL of fresh 1 N NaOH to aid solution, filter-sterilize, and store at room temperature. Prepare thymidine as a 500X stock by dissolving 302.5 mg thymidine (Sigma) in 50 mL of H₂O; filter-sterilize and store at 4°C. Prepare amethopterin (methotrexate) as a 500× stock by dissolving 11.4 mg of amethopterin (Sigma) in 25 mL of HBSS; filter-sterilize and freeze in small aliquots (light-sensitive).

3. Methods

3.1. Preparation of Targeting Vectors for Electroporation

Targeting vector DNAs are prepared for electroporation in an identical manner whether they are gene knockout vectors or *APRT*-targeting vectors. For gene knockout experiments, the targeting vector is linearized at a unique restriction site, such as *ScaI* in the plasmid backbone (i.e., opposite plasmid homologous *ERCCI* DNA sequence containing *NEO* and flanked by HSV-*tk* sequences; see **Fig. 1**). For *APRT*-targeted recombination assays utilizing an insertion-type targeting vector configuration, the pAG100 targeting is linearized by cutting at a unique restriction site within the region of *APRT*-targeting sequence homology, such as the *EcoRI* restriction site as shown in **Fig. 2** (top left). For *APRT*-targeted recombination assays utilizing replacement-type targeting vector configurations, the pAG100 targeting vector is linearized by cutting at a unique restriction site at either the 5'- (*SacI*) or 3'- (*BamHI*) end of the plasmid *APRT* sequence (see **Fig. 2**, top middle and right).

1. Linearize the vector by overnight restriction endonuclease digestion of plasmid DNA, using 2–3 U of restriction enzyme/ μ g of DNA, and employing the specific reaction buffers and conditions recommended by suppliers.
2. Treat the linearized targeting vector DNA with fresh proteinase K (200- μ g/mL) in 0.1% SDS, 1 M lithium acetate for 2 h at 50°C.
3. Purify the DNA by one phenol extraction (equal volume) using equilibrated phenol warmed to room temperature.
4. Recover the DNA from the separated aqueous phase by adding 2 vol of absolute ethanol, chilling on ice, and centrifuging in a microcentrifuge.
5. After rinsing with ice-cold 70% ethanol and air-drying for 20–30 min, resuspend the pellet in TE buffer overnight at 4°C to achieve a DNA concentration of 250–500 μ g/mL. Store frozen. (See **Note 9**.)

3.2. Growth and Preparation of Cell Cultures

CHO cell cultures are grown and prepared for electroporation in an identical manner whether targeted gene knockout or *APRT*-targeted recombination assays are to be performed.

1. Harvest cells from exponentially growing (~50–60% confluent) monolayer cultures of CHO cells grown in T-150 flasks (Corning, NY) at 37°C in a humidified atmosphere of 5%CO₂/95% air. Rinse the cell monolayers twice with prewarmed, sterile solution A, and expose to 2 mL of trypsin-EDTA, which is aspirated and discarded after 60–90 s. Tap the flasks forcefully several times, and resuspend the detached cells in 10 mL of ice-cold α MED–10% FCS by vigorous and repeated pipeting. After low-speed centrifugation, put the cell pellets on ice, and use within 15–20 min (see **Note 10**).

2. Based on cell counts obtained using a Coulter (Hialeah, FL) Counter, determine the volume of a cell suspension to spin down in a 15-mL sterile conical polypropylene centrifuge tube in order to provide sufficient cells for one electroporation (1×10^7 cells/cuvet for knockout electroporations; 2×10^7 cells for *APRT*-targeted recombination experiments). (See **Note 11**.)
3. Using a sterile, plastic 1-mL pipet, aseptically resuspend each cell pellet in 0.9 mL of sterile, ice-cold, electroporation buffer (HEP for targeted knockout experiments; LEP for *APRT*-targeted recombination experiments) containing restriction enzyme-linearized targeting vector DNA. For gene knockout experiments, a final concentration of 1×10^7 cells and 20 μ g of DNA/mL of HEP buffer should be achieved; for *APRT*-targeted recombination experiments, a final concentration of 2×10^7 cells and 10 μ g DNA/mL of LEP buffer should be achieved. (See **Note 12**.)
4. Using a sterile, plastic 1-mL pipet, immediately transfer 0.85 mL of the CHO cell/targeting vector DNA suspension in electroporation buffer to a prechilled, sterile electroporation cuvet (Bio-Rad, 4-mm electrode gap) and place on ice for a 10-min, pre-electroporation incubation.
5. Electroporate by delivering a single pulse of 500 V at 25 μ F to the cuvet, using a Bio-Rad Gene Pulser apparatus, and immediately place the cuvet back on ice for a 10-min, postelectroporation incubation. (See **Note 13**.)
6. Using a sterile plastic pipet, dilute the electroporated cell suspension by pipeting the contents of the cuvet into 8.5 mL of cold α MEM–10% FCS (also add one 1-mL rinse of the cuvet with α MEM–10% FCS), yielding a final cell concentration of $\sim 1\text{--}2 \times 10^6$ cells/mL.
7. For gene knockout experiments, make a further dilution with ice-cold SFMEM to yield a final volume of 60 mL. Plate 0.2 mL of this suspension to each of 300 100-mm tissue-culture dishes that contain 10 mL of α MEM–10% DFCS. Incubate the dishes at 37°C for 16–24 h in a humidified atmosphere of 5% CO₂/95% air. (See **Note 14**.)
8. For *APRT*-targeted recombination experiments, plate the resuspended cells into six 100-mm tissue-culture dishes, each containing 10 mL of α MEM–10% FCS. Incubate the dishes at 37°C for ~ 48 h in a humidified atmosphere of 5% CO₂/95% air before replating into selection medium. (See **Note 15**.)

3.3. Selection and Screening of Targeted Gene Knockout Clones

1. After 24 h, add G418 to each of the 300 dishes to achieve a final concentration of 400 μ g/mL (adjusted to 100% activity). After an additional 24 h of incubation, add FIAU to a final concentration of 0.2 μ M to all except 16 plates. Refeed the dishes with α MEM–10% DFCS containing G418 and FIAU (except the 16 dishes with G418 alone) every 3–4 d until visible colonies are evident, approximately 12–14 d postelectroporation.
2. Pick well-isolated G418 /FIAU-resistant colonies by gentle scraping with a Pipetman P-200 while pipeting up 200 μ L of culture medium using sterile, wide-bore yellow tips. To ensure that cell clones are independent, pick only 1 colony/

- dish. For each set of six picked and resuspended colonies, inoculate two six-well dishes: 50 μ L/well to a reserved master dish, and 150 μ L to wells of a corresponding dish to be used for DNA isolation for screening by PCR.
3. Stain the plates after marking the picked colonies with a felt pen on the bottom of the dishes; enrichment can be calculated by counting the total number of colonies from the 16 plates that were subjected to G418 selection alone, normalizing to a per-plate value, and comparing to the number of colonies per plate from the G418/FIAU-selected cells.
 4. After 3–5 d, cell monolayers growing in all wells of six-well DNA dishes are rinsed twice with solution A. Treat with trypsin–EDTA for 1 min. Aspirate the trypsin solution and discard. Inactivate the trypsin by adding α MEM–10% FCS to the wells, and resuspend the cells by vigorous pipeting. Pool cell suspensions for each dish into one tube (Falcon 1063, Becton Dickinson, Franklin Lakes, NJ), harvest by low-speed centrifugation, wash once with PBS, and resuspend in 50 μ L of PBS. Lyse the cells by adding 200 μ L of H₂O and heating for 10 min at 93°C. To the cell lysate, add 10 μ L of fresh 1% proteinase K, incubate for 30 min at 55°C, heat for 10 additional minutes at 93°C to inactivate the proteinase K, centrifuge, and aspirate the supernate for PCR analysis.
 5. The resulting lysate is used in a two-step nested PCR strategy as shown in **Fig. 1**. A 5'-primer (Forward Neo 1: 5'-CCGCTTTTCTGGATTCATCGAC-3') designed to anneal to *NEO* sequence within the targeting vector, and a 3'-primer (Reverse ERCC1: 5'-TGCCACCCCTGACCACATATACAC-3') designed using *ERCC1* sequence downstream from exon V and not present in the targeting vector are used with 5 μ L of cell lysate from the previous step in a 50- μ L total reaction volume containing 1 μ L *Taq* Extender (Statagene) and 1 μ L *Taq*Start Antibody (Clontech). Perform 20 cycles of PCR (1.5 min at 95°C, 1 min at 56°C, 3 min at 73°C). Perform a second round PCR with 5 μ L of first round amplicon using a nested *NEO* primer (Forward Neo 2: 5'-GCTTCCTCGTGCTTTACGGTATC-3'). After electrophoresis on 0.6% agarose gels, identify pooled colony samples exhibiting a diagnostic 1.8 kb targeting fragment (see **Fig. 1**).
 6. To verify successful gene targeting, individually trypsinize and transfer to T-25 flasks the six cultures from each reserved master dish corresponding to a positive pooled sample. Repeat the PCR analysis as described in **step 5** to identify which of the six clones exhibits the diagnostic recombinant fragment. Southern analysis (see **Subheading 3.5., below**) can be used to confirm disruption of the target gene by the *NEO* sequence present in the knockout vector.

3.4. Selection of APRT-Targeted Recombinant Clones

1. Approximately 48 h after electroporation, trypsinize and suspend the cells from each 100-mm dish, centrifuge, and resuspend in 10 mL of ice-cold α MEM–10% FCS. (See **Note 16**.)
2. Take a small aliquot from each suspended cell population to count, serially dilute, and plate into α MEM–10% FCS medium for plating efficiency (PE) determinations (six 60-mm dishes at ~200 cells/dish). Colonies arising on PE dishes can be

- fixed, stained, and counted after ~8 d of incubation.
3. Plate a small portion of the resuspended cells from each 100-mm dish into HAT selection medium (three 100-mm dishes at 150 μ L of cell suspension/dish) to select for GPT⁺ nontargeted vector integration events and monitor electroporation efficiency. Colonies arising on HAT selection dishes can be fixed, stained, and counted after ~12 d of incubation.
 4. Plate the remainder of the cells from each 100-mm dish into ALASA selection medium (six 100-mm dishes at 1.5 mL of cell suspension/dish) to select for APRT⁺ recombinants. Representative ALASA^r APRT⁺ recombinant clones can be picked for analysis after ~14 d of incubation (*see Note 17*), by gently scraping off and pipeting up cells from individual colonies using sterile, wide-bore yellow tips and a Pipetman P-200, as described in **step 2** of **Subheading 3.3**. The rest of the colonies can then be fixed, stained, and counted.

3.5. Analysis of APRT-Targeted Recombinant Clones

1. Isolate genomic DNAs from each independent ALASA^r recombinant clone, as well as from the ATS49tg cell line.
2. Subject DNA samples from each clone (and from ATS49tg) to overnight restriction endonuclease digestion by *Mbo*II or *Hind*III, using 2–3 U of restriction enzyme/ μ g of DNA, employing the specific reaction buffers/conditions recommended by suppliers.
3. Following restriction endonuclease digestion and electrophoresis (560 volt-h) in 0.8% agarose gels, transfer by capillary blotting to nitrocellulose or nylon membranes. Probe the blot with nick-translated or random-primed, ³²P-labeled *APRT* gene-specific probes. For *Mbo*II digests, a 1.4-kb *Eco*RI-*Xba*I *APRT* fragment is used. For *Hind*III digests, two different probes are used: a 0.6-kb, 5'-end, target gene sequence-specific (*Bam*HI-*Eco*RV *APRT* fragment) probe, and a 3.9-kb *Bam*HI fragment that includes the entire CHO *APRT* gene sequence.
4. For Southern blotting hybridization and washing conditions, supplier's instructions for radioactive hybridization (Stratagene QuikHyb Instruction Manual, pp. 2–4) can be followed without modification.
5. The various classes of targeted *APRT* recombinants (**Fig. 2A,B,C**) can be readily identified on the basis of their distinctive and diagnostic restriction fragment patterns. (*See Note 18.*)

4. Notes

1. CHO-ATS49tg is hemizygous for the *ERCC1* gene (**14,18**). For *ERCC1* gene knockout experiments as described in this chapter, we have used CHO-K1 cells as well as several other CHO cell lines, including ATS49tg, derived from CHO-AT3-2 cells (**37**).
2. Construction of the *ERCC1*-targeting vector pSL1 is described in ref. (**14**). In principle, a repair gene targeting vector with the replacement-type structure illustrated in **Fig. 1** could be constructed using *NEO* and *HSV-tk* markers and used to knockout a variety of NER or DSB repair genes in CHO or other cultured mammalian cell lines.

3. Some positive–negative selection gene-targeting protocols use FIAU in selection medium containing nondialyzed serum; however, we find that FIAU counter selection is more stringent and economical (requiring much less FIAU) if dialyzed serum is used.
4. These Forward Neo PCR primers, nested in the *NEO* cassette from plasmid pMC1neopA (36), should be useful for any PCR screening strategy in which *NEO* is used as the gene-disruption marker and the reverse PCR primer is designed from target gene sequence outside the homologous sequence in the targeting vector, and downstream (3') from the exon to be disrupted.
5. CHO-ATS49tg is an *APRT*⁻/*HPRT*⁻ CHO cell line that is hemizygous for the *APRT* locus (28). CHO-ATS49tg cells contain a single, mutationally altered copy of the *APRT* gene, with a 3-bp deletion in exon 5 that has eliminated an *Mbo*II restriction site (28,38), providing a convenient restriction fragment length polymorphism for monitoring whether targeted recombination events have resulted in correction of the *APRT* target gene defect. We have also used other CHO cell lines derived from CHO-AT3-2 with hemizygous, mutant *APRT* alleles suitable for targeted correction in gene-targeting experiments (12,12a,39).
6. Plasmid pAG100 (12), which contains ~3.2-kb of 5'-truncated *APRT*-targeting homology, along with a *GPT* gene cassette driven by an SV40 promoter (see Fig. 2), was derived from pSV2gpt (40) in several steps that involved the construction of intermediate plasmids, using conventional cloning methods. First, the plasmid, pAG7 (28), was constructed by replacing the 0.7-kb *Eco*RI-*Bam*HI region of pSV2gpt with a 2.6-kb *Eco*RI-*Bam*HI fragment derived from pHprt-1 (41), containing the 3'-portion of the Chinese hamster *APRT* gene. Plasmid pAG1 (30) was then derived from pAG7 by inserting a 1.3-kb *Eco*RI fragment containing the promoter region and 5'-portion of the *APRT* gene into the pAG7 *Eco*RI site to reconstruct a complete, functional *APRT* gene. Plasmid pAG100 was derived from pAG1 by excision of the 0.6-kb *Sma*I-*Eco*RV region containing the promoter and first exon of the *APRT* gene, and ligation of the blunt ends to recircularize the plasmid.
7. Alanosine and azaserine act as inhibitors of different steps in the *de novo* pathway for purine biosynthesis. *APRT* is a purine salvage pathway enzyme that allows cells to utilize adenine for purine biosynthesis by catalyzing the conversion of adenine to AMP. ALASA selection medium will effectively kill *APRT*⁻ cells, such as CHO-ATS49tg, which are unable to utilize exogenous adenine as a purine source, but will support the growth of *APRT*⁺ recombinants.
8. Amethopterin (methotrexate) acts as an inhibitor of steps in the *de novo* pathways for both purine and pyrimidine biosynthesis. Both *HPRT* and its bacterial equivalent, *GPT*, are purine salvage pathway enzymes that allow cells to utilize hypoxanthine or guanine for purine biosynthesis. HAT selection medium will effectively kill *HPRT*⁻ cells, such as CHO-ATS49tg, which are unable to utilize exogenous hypoxanthine as a purine source, but will support the growth of *GPT*⁺ transfectants.

9. All steps subsequent to ethanol precipitation should be performed aseptically in a tissue-culture hood. DNA pellets may be left to air-dry for 20 min in a sterile hood, and then resuspended using sterile technique
10. To achieve maximum and reproducible transformation efficiency in gene-targeting experiments, it is important that the cells are in exponential growth at the time cell cultures are trypsinized and harvested for electroporation.
11. Cells should be harvested such that only one cell pellet of $1-2 \times 10^7$ /centrifuge tube is used for one cuvet; we have experienced poor results with attempting to resuspend sufficient cells for several cuvetts in a single centrifuge tube, and then distributing the suspension to multiple cuvetts.
12. It is important to aspirate the supernatant fluid as completely as possible after centrifugation. Prior to resuspending cells in electroporation buffer, strike the conical centrifuge tube containing the cell pellet sharply several times against the tissue-culture hood surface to loosen the pellet. Then, resuspend the cells very carefully by pipeting back and forth slowly several times, without introducing bubbles. For *APRT*-targeted recombination experiments, it is important that the cell pellet be directly suspended in LEP buffer (without a rinse step) after carefully draining off the supernatant and aspirating any remaining traces of liquid from the sides of the tube. Inclusion of an LEP buffer rinse step reduces cell survival after electroporation; this procedure is also followed for knockout gene-targeting experiments using HEP buffer.
13. For knockout electroporation in HEP buffer, time constants in these conditions should be ~ 0.6 . For *APRT* gene targeting in LEP buffer, time constants in these conditions should be $\sim 5.5-6.0$. The 10-min postelectroporation incubation on ice is not necessary for knockout electroporations in HEP buffer.
14. One or two cuvetts are a standard size experiment for knockout gene targeting, resulting in 300–600 dishes. In our experience, one cuvet will produce approx 180–200 G418/FIAU doubly resistant colonies after positive–negative selection.
15. For *APRT*-targeting experiments, a standard-size experiment assessing the effects of one targeting vector structure on targeted homologous recombination in one cell line is four or six cuvetts, resulting in 24–36 dishes prior to plating into ALASA selection medium. Two or more cell lines, and one or two targeting vector configurations are usually assayed in an experiment.
16. It is important to:
 - a. Keep this cell suspension (and any dilutions to be used for plating) on ice to minimize cell clumping or attachment.
 - b. Make sure that the cells are kept well suspended throughout the plating procedure, and that the cells are uniformly distributed after plating into tissue-culture dishes.
17. To ensure the independence of all *APRT*⁺ recombinants analyzed, only a single ALASA^r colony from each independent cell population should be picked for expansion, cryogenic preservation, and Southern blot analysis.

18. $APRT^+$ recombinants that have arisen by target gene conversion (**Fig. 2A**) should show the loss of the 2.0-kb *ATS49tg MboII* fragment and the reappearance of a 1.5-kb *MboII* fragment characteristic of a wild-type Chinese hamster *APRT* gene, but should retain normal, wild-type *APRT* restriction fragment patterns for all other restriction enzymes, including *HindIII* (which yields a single 8.6-kb restriction fragment). Recombinants that have arisen by targeted insertion (**Fig. 2B**) should show diagnostic 10.3- and 6.0-kb *HindIII* restriction fragments after hybridization with the 3.9-kb *BamHI* fragment probe; only the 10.3-kb fragment will hybridize with the 5'-end-specific *BamHI-EcoRV* fragment probe. Two subclasses of targeted insertions may be distinguished after *MboII* digestion and hybridization with the 1.4-kb *EcoRI-XbaI* fragment probe: Type I (*MboII* +/ Δ) targeted insertions, in which the downstream 5'-truncated copy of the *APRT* gene retains the original *ATS49tg* 3-bp exon 5 *MboII* site deletion, and Type II (*MboII* +/-) targeted insertions, in which both copies of the *APRT* gene have a wild-type exon 5 *MboII* site. Type I (*MboII* +/ Δ) targeted insertions will show both a 1.5-kb (wild-type) and 2.0-kb (mutant) *MboII* fragment, whereas type II (*MboII* +/-) targeted insertions will show only a 1.5-kb (wild-type) *MboII* fragment. $APRT^+$ recombinants that have arisen by target gene-templated extension and correction of the pAG100 targeting vector *APRT* sequence (**Fig. 2C**) should thus contain both the original 2.0-kb (mutant) *MboII* fragment and a newly acquired 1.5-kb *MboII* fragment characteristic of a wild-type CHO *APRT* gene. After digestion with *HindIII* and hybridization with the 5'-end, target gene sequence-specific *BamHI-EcoRV APRT* probe, such vector correction recombinants should show two distinct hybridizing fragments; an 8.6-kb fragment characteristic of the endogenous *ATS49tg APRT* locus, plus a novel fragment of indeterminate size that represents the randomly integrated, wild-type recombinant *APRT* gene.

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Measurement of Low-Frequency DNA Breaks Using Nucleoid Flow Cytometry

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1. Introduction

The organization of eukaryotic DNA is increasingly well understood at each extreme of chromatin organization. Advances in understanding the organization of DNA at the level of nucleosome and chromosome have outstripped knowledge of the intermediate states and function of DNA. Most would, however, agree on the following general hierarchy (1,2). The base-repeating unit of approx 146 bp, including a core histone group, is further organized into the 30-nm fiber and constrained by contact with the nuclear matrix to form loop and superloop structures capable of maintaining physical tension or supercoiling (3–5). These supercoiled units of DNA subsequently adopt repetitive patterns based around a protein core template, which together are observed as chromosomes.

Nucleoids, sometimes referred to as histone-free nuclei, are formed after the extraction of most proteins and histones, leaving the organization of repetitive supercoiled DNA intact. Ethidium bromide, by intercalating within the DNA helix, can readily relax native DNA supercoiling (–ve by convention) to a zero point of no supercoiling and then by continuing to exert a twisting force, impose +ve supercoiling. This physically results in an expansion and then contraction of nucleoid size. Breaks within the DNA strand permit the relaxation, or loss of supercoiling, within individual loops, which then extends to much larger structures or domains, which may exceed 1 Mbp in size (6). Thus, nucleoids containing DNA damage are physically larger than controls and deflect more light when passed through a flow cytometer. It is this amplification of a single-strand break into an effect on such large tracts of DNA that gives this technique its sensitivity and, in the form discussed here, rapidity of analysis. Using single-

cell suspensions, data collection may be started approx 90 s after treatment of viable cells.

It should be made clear, however, that in measuring DNA damage with this technique an extra dimension is added over and above that seen after intercalator-induced changes in supercoiling. The nucleoid response to DNA damage can be considered as affected by two levels of organization—DNA loops bounded by weak links and topologically closed DNA domains bounded by strong links. Ethidium bromide-driven relaxation and rewinding can be explained by reference to changes within the DNA loops bounded by the numerous weak DNA links, whereas DNA breaks disrupt multiple weak links, propagating DNA unwinding from the site of the DNA lesion, and are only contained by the strong boundaries of the topological domains. Thus, a topologically relaxed domain that contains a DNA break may still be subject to manipulation of the embedded loops of DNA by ethidium bromide. This feature has implications in the measurement of DNA damage and will be further discussed in **Subheading 3**.

Measurement of the presence of damaged DNA is achieved by detecting the forward light-scatter signal within a flow cytometer from the relaxed DNA domains that have lost their intrinsic supercoiling. Nucleoids containing DNA breaks are therefore physically larger than those from undamaged cells and generate a DNA damage-dependent increase in the light-scatter response.

2. Materials

1. Cytometer: Most experience has been gained with Becton Dickinson (San Jose, CA) FACS type (FACS 420/440/FACStar) research cytometers offering “Jet in air” sample interaction. However, with little or no modification, the more routinely available, and substantially cheaper, FACScan devices, and systems with similar technology from other manufacturers may also be used.
2. Basic nucleoid lysis buffer: 2 M NaCl, 10 mM Tris-HCl, 10 mM EDTA, 0.1% Triton X-100, and from 0–20 µg/mL ethidium bromide adjusted to pH 8.3.
3. Fluorescent-capable microscope for checking structures.

3. Methods

3.1. Setup—Biology

Prepare viable cell cultures in asynchronous or synchronized growth as required (*see Note 1*). During and after nucleoid preparation, all manipulations should be on ice. Prepare cells, from 10^5 – 10^6 /sample, as a pellet in the machine sample tube itself or in a smaller tube inside the main tube. Prior to the addition of lysis buffer, resuspend the cell pellet in residual buffer with vigorous hand or vortex mixing. The addition of 0.2–1.0 mL of lysis buffer

will rupture cell and nuclear membranes, and remove all histones and most proteins. This and all subsequent steps should be carried out at 0–4°C. The residual structure will contain naked DNA arranged in loops both within and exterior to the nuclear matrix. As might be imagined, such structures are fragile. For this reason, the lysis step is critical. Addition of the buffer should be done rapidly using a pipet. To mix the preparation, gently swirl the solution while holding the tube firmly at the top. Ethidium bromide stain may be added with the lysis buffer or after. If added after, and sufficient stain is used, adequate mixing can be readily observed by the even red color produced. **Do not** use a vortex mixer once the cells are lysed, or over shake the sample, this will rapidly shear fragile DNA structures. Stain two separate samples to a final concentration of 5–7 and 20 $\mu\text{g/mL}$ of ethidium bromide, and store the tubes on ice. The lower concentration will, by intercalation into the DNA double helix, produce an unwinding of the native negative supercoiling leading to nucleoids containing loops that contain no or few supercoils. In practice, a slight adjustment over the range quoted will give maximal relaxation, but within this range, all eukaryote cells so far studied will contain more relaxed DNA domains than those stained with the higher concentration of ethidium bromide. This should be observed microscopically. What will be seen at the lower concentration are fluorescent objects with a fuzzy halo, which are the relaxed and extruded loop structures, surrounding a central core. The higher concentration—in addition to being brighter owing to the increased fluorescence—will not show any detectable halo. This extremely quick check of the biological mechanics of the system is invaluable as a setup tool for cytometry studies and as a quick reference for correct system operation should problems arise (*see* **Notes 2** and **3**). If required, a complete ethidium bromide titration curve may be produced showing the dose-dependent change from -ve to zero to +ve DNA supercoiling (**Fig. 1**).

3.2. Setup —Cytometry

This discussion assumes a basic knowledge of the operation of a flow cytometer instrument.

1. The machine should be set to deliver a 488-nm laser line.
2. High power is not normally required; 100 mW is sufficient for most studies. If a choice of nozzle sizes is available, 70 μM has proven suitable. If in doubt, use a larger nozzle than this to avoid the production of clogs in the nozzle orifice.
3. All regular schedules for conventional cytometer set up using beads, cell cycles from established lines, chicken red blood cells, and so forth, have the common goal of aligning both the optical and particle paths, such that an exact and reproducible orthogonal alignment of beam and sample path is produced. This is essential for correct analysis of nucleoids, and once set should not be changed.

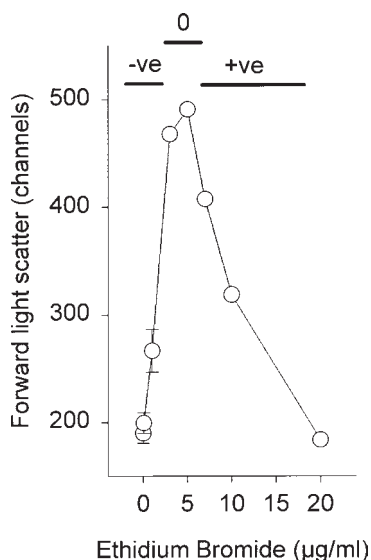


Fig. 1. Ethidium bromide titration curve showing that on addition of increasing amounts of the DNA intercalator ethidium bromide, alteration from negative (native) through zero to positive supercoiling. Such a response to ethidium bromide is only possible within closed, supercoiled loops of DNA.

3.3. Setup—Data Collection

1. For machine setup, the nucleoids stained with 5–7 and 20 µg/mL ethidium bromide should be used. These are stable for a period of hours depending on the cell line. However, it is recommended that fresh samples be prepared often because some cell types may become unstable or coagulate over time.
2. Accumulation of data should be made triggering on red (DNA) fluorescence, and data collection should be a dual plot of DNA fluorescence against forward scatter. It may be beneficial to operate the red (DNA) channel in log scale to compress the signal. This will simplify collection of data if variable amounts of ethidium bromide are used (**Fig. 1**). This will also ensure that data will be recorded from nuclear (stained with ethidium bromide) material and reduces the possibility of collecting information from debris. Subsequently, observation of the scatter histogram data alone may be more convenient for following the progress of an experiment (*see Notes 4 and 5*).
3. If the cytometer used has optional analog-to-digital conversion methods, the default setting should be pulse-height conversion (*see Note 6*).
4. It may be necessary to alter the settings of the forward-scatter collection device. Nucleoid structures are larger than cells or nuclei preparations and may require the addition of a neutral density filter (which reduces signal intensity by some

- factor, usually 90%) and/or gain setting changes of the CCD or photomultiplier detector to keep the signals on scale.
5. Machine settings specific for the collection of nucleoid data are restricted to careful alignment of the forward-scatter obscurator bar. The purpose of this bar is to block the full laser beam from entering the forward scatter detector and saturating the data collection system. The scatter signals coming from irradiated nucleoids will include low-angle events, and to ensure these are observed, the scatter bar must be placed to offer the smallest profile to the beam. This may be best achieved by using very small beads ($\sim 2 \mu\text{m}$) to optimize blocking of the beam with collection of a scatter signal.
 6. At least 10,000 events should be recorded using a flow rate of approx 50–200 events/s. It is possible to analyze intact ethidium bromide-stained nucleoids at a higher rate than this, but most test systems containing damaged DNA are fragile and require more gentle handling.
 7. Running stained nucleoids through the system will generate scatter signals of higher amplitude for nucleoids stained with the low concentration, decreasing at the higher concentration of ethidium bromide as the loops rewind. The data from a single histogram will be observed as a broad Gaussian-like distribution. The mean of this distribution is the simplest way of numerically analyzing and comparing such data (**Fig. 2**).
 8. As a guide, a minimum of a twofold difference in mean nucleoid scatter should be observed between nucleoids containing relaxed or condensed DNA loops induced by the low and high concentrations of ethidium bromide. This setup protocol should be carried through every time a nucleoid analysis is done. It serves a dual purpose in that it confirms the machine is detecting alterations in nuclear DNA supercoiling and sets the low and high forward scatter parameters for subsequent data collection. The majority of nucleoid DNA damage, and its repair that may be observed will fall between these two scatter end points.

3.4. Assay of DNA Damage

The following discussion relates to the measurement and repair of DNA damage introduced by irradiation (**Fig. 3**). This is perhaps the simplest system to study in terms of DNA damage, though of course other damaging agents have and could be assessed (*see Note 7*). Simplistically the system requires only running samples through the machine and recording the mean light scatter. However a number of factors will affect the desired outcome.

1. As discussed in **Subheading 1.**, the relaxation of supercoiled domains by DNA breaks differs from changes induced by ethidium bromide. What this means is that the measurement of topological relaxation is not instantaneous—which is effectively the case for ethidium bromide-induced changes—but requires a time period for equilibrium to occur. This equilibrium is a composite of events which are driving relaxation (primarily the loss of DNA integrity) countered by events that resist relaxation, such as the ethidium bromide-induced contraction of loop

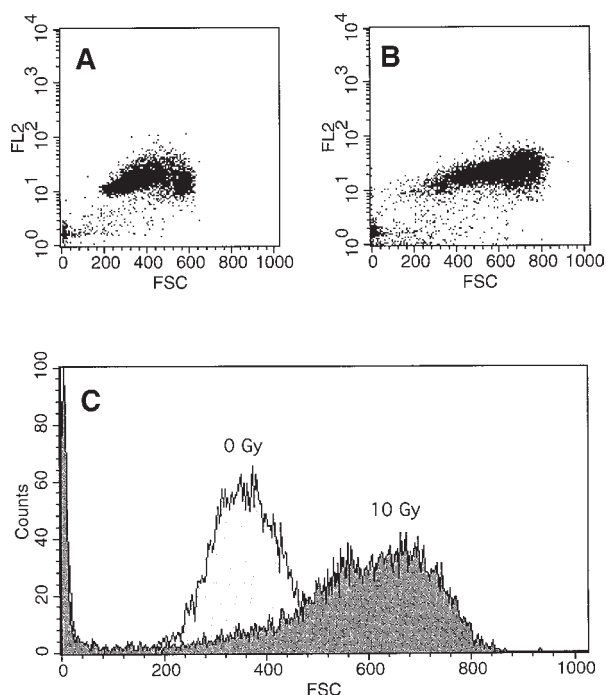


Fig. 2. Collection of raw data from nucleoids extracted from human SQ-20B tumor cells both with and without irradiation on ice prior to nucleoid extraction and analysis, a trace amount (0.5 $\mu\text{g/mL}$) of ethidium bromide is used to identify the nucleoids. Panel **A**, a forward scatter (FSC) and DNA fluorescence (FL2) dual-parameter plot of 10,000 individual nucleoid events taken from an unirradiated population. Panel **B**, the same number of nucleoid events as panel **A**, but obtained immediately after irradiation of cells with 10 Gy on ice. No time for repair was allowed. Panel **C**, overlaid histograms of the FSC events produced in panels **A** and **B** showing the separation of the control and irradiated populations. If repair time is allowed the distribution of FSC events from nucleoids extracted from the irradiated population will decrease, as DNA breaks are rejoined and intact topological domains are reformed.

elements or the intrinsic resistance to propagation of loop unwinding from the site of a DNA break through “weak” loop attachments to the borders of the topological domain. In practice, this means that there is a delay in the nucleoid relaxation response reaching an equilibrium. To overcome or at least mitigate this problem, three strategies may be employed.

- Increase and standardize the lysis time prior to analysis. This will allow time for the system to approach an equilibrium prior to analysis.
- Increase or alter the lysis buffer contents, particularly in terms of detergent concentration or type. Higher concentrations of detergent may loosen

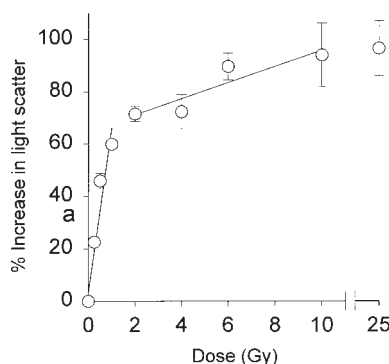


Fig. 3. Radiation dose-response of nucleoid relaxation carried out without any ethidium bromide. Note that half of the maximum relaxation is observed at a dose of 0.5 Gy.

DNA matrix attachment sites, again allowing a more rapid approach to equilibrium.

- c. Eliminate high concentrations of ethidium bromide. This removes the main antagonist to DNA break-driven topological relaxation, and thus, equilibrium is reached much more rapidly.

Of the three possibilities (none of which are mutually exclusive), the elimination or reduction of ethidium bromide content is perhaps the most attractive. This still allows trace tagging of nucleoids with 0.1–0.5 $\mu\text{g/mL}$ of ethidium bromide, which is sufficient to trigger data collection from the DNA fluorescence signal, but not enough to have a significant effect on DNA supercoiling (**Fig. 1**). In our hands, this has led to the most sensitive measurement of DNA damage in mammalian systems.

2. Nucleoids containing broken DNA are more likely to produce clumps in the sample tube and run more erratically through the machine. If this is a problem, make sure that the sample-collection tube is cleaned routinely using dilute bleach or similar agent. In extreme cases, the nozzle may have to be removed and cleaned in an ultrasound bath containing detergent or bleach solution. Normally, this will require a complete reset of the machine-operating parameters.

4. Notes

1. In the case of synchronized cells, it must be remembered that nuclear size does increase through the cell cycle, and this may lead to confusion unless appropriate controls are used.
2. If no difference in scatter signals is observed during set up, check the obscurator bar setting, and adjust for the smallest profile to the beam.
3. It has been observed that occasionally the ability to detect changes in nuclear supercoiling becomes very poor for no obvious reason. At this stage, a complete change of all buffers and reagents has worked. Nobody knows why.

4. The operation in dual-parameter mode allows a better visualization of the process. If analysis is pursued very rapidly from initial lysis—in a matter of seconds—it will be possible to observe the signal degradation that ensues from the loops spilling out from the central core. This will lead to an unavoidable loss in perceived data quality from what will seem to be a satisfactory cell-cycle profile. This is in fact a reasonable index of the success of the lysis procedure in that the lowering of the distribution CV is an indication that a suitable nucleoid preparation has been made.
5. It is possible to detect alterations in light scatter by recording data collected at right angles to the laser beam. This will certainly respond to alterations in nucleoid organization induced by DNA damage and so may be used, but its interpretation is not straightforward, because the situations that produce high-angle scatter within a translucent nucleoid structure are obscure.
6. Other A/D conversion settings will work, such as pulse width. However, nucleoid structures generate in general poorer and more erratic signals than defined particles, such as cells or beads, and pursuing electronic manipulation of such data must be attempted and viewed with caution.
7. Not all DNA damage is created equal. For example, exposure to epipodophyllin toxins, such as VP16, introduces DNA breaks by freezing the cleavable complex involving topoisomerase II. Such frank breaks are, however, only seen after digestion of the topoisomerase link to the DNA break. After simple nucleoid extraction, such lesions will maintain DNA linear integrity, and thus DNA supercoiling, and will not therefore generate an increase in nucleoid size.

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Live Analysis of the Division Cycles in X-Irradiated *Drosophila* Embryos

John Cunliff, Justin Blethrow, and William Sullivan

1. Introduction

Early *Drosophila* development begins as a syncytium with 13 rapid nuclear divisions without cytokinesis (1). These divisions provide an excellent system in which to study the effects of DNA damage on the mitotic cycle. Because the effects of ionizing radiation can be assessed in both wild-type and mutant backgrounds (i.e., mutagen-sensitive strains lacking DNA repair machinery), the early *Drosophila* embryo is a potent genetic system to assay these perturbations. In addition, the events of early embryogenesis have been characterized in detail through both fixed and live analysis (1–3).

In spite of these advantages, analysis of the effects of DNA damage during these early divisions has remained relatively unexplored. Only a few studies have examined the effects of X-irradiation on the syncytial divisions, and very little is known about the DNA repair and checkpoint control during these stages (4). In addition, although a large number of X-ray-sensitive mutations have been isolated, very few have been characterized with respect to their effects on the embryonic syncytial divisions (5).

Recent advances are changing this situation as researchers employ new techniques that make live analysis of X-irradiated embryos a straightforward procedure. Direct injection of fluorescently labeled proteins (such as histone, tubulin, or actin) or the use of transgenic flies that express Green Fluorescent Protein- (GFP) reporter constructs are excellent reagents to use in live analysis of the effects of X-irradiation on nuclear and cytoskeletal dynamics (2,3,6,7). As an example, **Fig. 1** depicts images from syncytial *Drosophila* embryos exposed to low doses of X-irradiation, injected with rhodamine-labeled his-

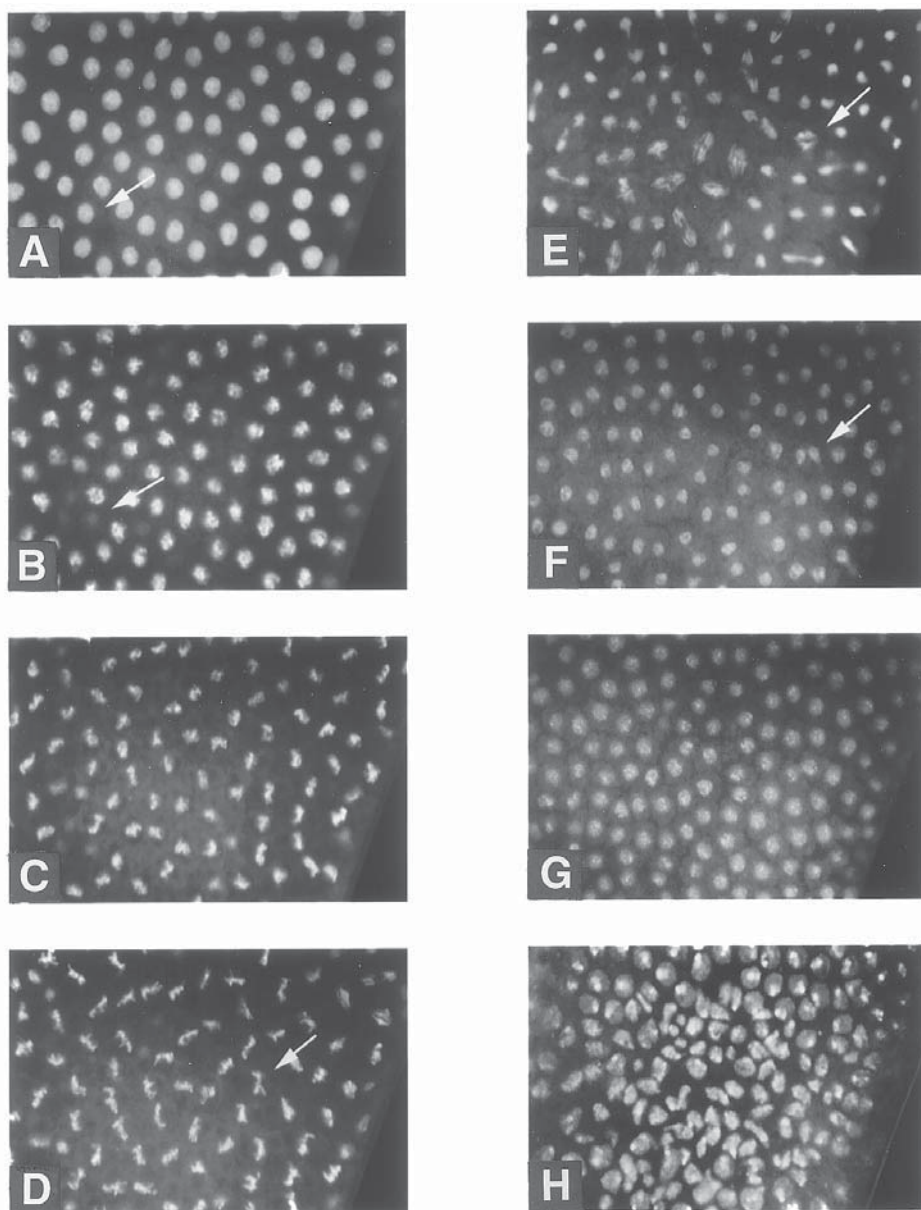


Fig. 1. Wild-type *Drosophila* embryos were injected with rhodamine-labeled histone and exposed to 300 rad of X-irradiation during interphase of cortical nuclear cycle 11. Panel A depicts the embryo 5 min after X-irradiation. Panel B depicts the embryo at 8.5 min after X-irradiation, and panels C, D, E, F, and G were taken at 3.5-min intervals. Panel H depicts the embryo 45 min after X-irradiation. The arrows in panels A and B follow damaged nuclei dropping into the interior of the embryo. The

tone, and visualized on a confocal microscope. Because of the rapid divisions, the effects of X-irradiation can be readily followed through multiple cycles.

This chapter describes a protocol for live analysis of X-irradiated embryos using the injection of fluorescently labeled proteins. If flies expressing GFP-reporter constructs are available, they may be used in place of or in combination with labeled protein injection. We outline a variety of imaging techniques for recording normal and aberrant divisions. In addition, difficulties, such as photobleaching, hypoxia, and hyperthermia, are discussed.

2. Materials

2.1. Embryo Preparation Materials

1. Mechanical needle puller.
2. Injection apparatus.
3. 75-mm Capillary tubing with an outer and inner diameter of 1.21 and 0.90 mm, respectively (catalog #N-51A, Drummond Scientific, Broomall, PA).
4. Microscope slides and 24 × 50 mm² cover slips (Fisher).
5. Drierite granular desiccant.
6. Halocarbon oil (Series 77, CAS no. 9002-83-9, Halocarbon Products Corp.).
7. 10-mL Hamilton syringe.
8. Double-stick tape (3M).

2.2. X-irradiation and Visualization

1. Inverted microscope with confocal imaging system.
2. X-ray source (Torrex X-ray generator available from Astrophysics Research, Long Beach, CA).
3. Fluorescently labeled protein: Molecular Probes (Eugene, OR) carries a wide variety of labeled proteins, including fluorescently conjugated bovine tubulin (catalog #T-7460).
4. GFP fly stocks (optional).

3. Methods

3.1. Advance Preparation

If you are using GFP embryos and do not plan to inject them, omit **steps 3 and 4**.

1. Concentrated embryo adhesive: Add approx 12 in. of double-stick tape to a 50-mL conical tube containing 10 mL of heptane and allow to rotate overnight. The resulting solution will contain suspended particulate and must be centrifuged to clarity. Aliquot the solution into 1.5-mL microcentrifuge tubes and centrifuge

arrows in panels **D**, **E**, and **F** follow damaged sister telophase nuclei snapping back and fusing. For a more extensive description of these phenotypes, *see* refs. (8) and (9).

- at 14,000 rpm (15,800g) for 15 min or until the solution is clear. Pool the resulting solution, and keep in a tightly closed container to minimize evaporation of the heptane. Make a working solution by diluting the concentrate roughly five to one. The adhesive is optimally diluted when evaporation of the heptane leaves a barely visible film on a cover slip.
2. Embryo preparation slides: Affix a 24 × 50 mm² cover slip to the sample area of a standard microscope slide with minimal tape. Place a small piece of double-stick tape at the frosted end of the slide. Draw 2–4 µL of diluted embryo adhesive in a vertical line along the middle of the cover slip. After the heptane evaporates, delineate the edges of the remaining adhesive with a marker. Prepare several slides in advance, and place in a closed container to minimize dust accumulation.
 3. Injection needles: Draw out three or four needles, and examine under a microscope. Choose for injection those that form a smooth unbroken tip. Store on a clay mount in a Petri dish at 4°C.
 4. Needle preparation slide: Break a microscope slide in half, and tape it onto a second slide with the unbroken edge facing inward. This edge will be used to break the tip of the needle. Place a small amount of halocarbon oil on the unbroken edge, and store the slide in a covered place.

3.2. Preparation and Injection of Embryos

1. Collect embryos over 30-min intervals at 25°C; collections that produce <15 embryos should be discarded. Age productive collections for an additional 30 min at 25°C. This results in the majority of the embryos being at approximately nuclear cycle 8 at the time of injection. If you are not planning to inject (i.e., if you are analyzing only GFP-tagged proteins expressed from transgenes), age the embryos for a total of 80 min. Following aging, collect the embryos, and place in small piles on the double-stick tape end of an embryo preparation slide. Dechorionate the embryos by gentle manipulation with an embryo pick (dissecting forceps), and then transfer to the cover slip. Be gentle when placing embryos on the cover slip, since they are easily damaged. Transfer a maximum of 10 embryos, leaving at least 3 mm between the embryos and the edge of the cover slip. If you will not be injecting, cover the embryos with halocarbon oil immediately to prevent desiccation, and proceed to **step 6**.
2. Desiccate the embryos by placing the slide in a drying chamber for 8–15 min. (A sealed plastic dish containing drierite serves as an effective drying chamber.) The drying time changes owing to day-to-day variation in temperature and humidity. Use the first collection to determine an appropriate drying time. Flacidity and indentations along the embryo are signs of overdrying. One must be careful to not to overdry the embryos, because this will create recording artifacts. Underdried embryos will bleed cytoplasm when injected.
3. While the embryos are desiccating, prepare the needle for injection. Thaw an aliquot of labeled protein and centrifuge for 3 min at 10,000 rpm (8000g). This pellets denatured or aggregated protein, and reduces the chance of the needle clogging. Use a 10-mL Hamilton syringe precooled to 4°C to backload the needle.

- Flick the needle forcefully to bring the fluid to the point. The loaded needle should be maintained in the dark at 4°C as much as possible.
4. The most technically demanding aspect of the injection procedure is generating a small hole, approx 3–5 mm in diameter, at the tip of the needle. For reference, syncytial interphase nuclei are approx 5 mm in diameter. Place the needle preparation slide on the stage, and bring the edge of the broken slide into view. Mount the needle in the microinjection apparatus and gently pressurize it. While maintaining pressure, slowly bring the needle into contact with the edge of the broken slide. A small break is readily detected, because fluid immediately flows from the needle and is clearly visible in the oil. Once this is achieved, remove the needle from the injection apparatus and store in the dark at 4°C. Kept in this manner, the loaded needle will suffice for a satisfying day of microinjection.
 5. Immediately after the embryos are dried, they are covered with halocarbon oil and microinjected. The most critical aspect of manual microinjection is needle pressure. To obtain the correct needle pressure, place the tip of the needle in the halocarbon oil near the embryos, and apply pressure until a small bubble of injection fluid is observed in the oil. Adjust the pressure until the bubble undergoes a steady, gradual increase in volume, and then pull the needle away from the bubble. If the pressure is correct, no fluid flows from the needle. When the needle is reintroduced into the bubble, fluid again flows, and the bubble increases in volume. With this pressure, the injected embryos absorb enough fluid to replace that lost by dehydration. Using this procedure, we typically inject approx 1–5% egg volume with fluorescently conjugated protein.
 6. After injection, remove the cover slip from the slide and place on the confocal microscope. Place a thermometer as close to the cover slip as possible, and record the ambient temperature (*see Note 1*). The labeled protein should be immediately visible in the interior of the embryo, but will take a cycle or two to reach maximum incorporation. Take a few minutes to find a minimally damaged embryo of the appropriate age on which to focus your attention. When time constraints allow, we find it useful to document the chosen embryo's development for a cycle or so prior to X-irradiation.

3.3. X-Irradiation

When the chosen embryo is of the appropriate age, capture an image as a control, and then quickly transfer the cover slip to your X-ray source and X-irradiate. The appropriate dosage will vary depending on the desired effect. A good starting dosage is 300 rad (**8,10**). This dosage will induce mitotic failure in the majority of nuclei and thus serves to illustrate the phenotype (*see Note 2*).

3.4. Microscopy and Video Production

1. Following X-irradiation, return the cover slip to the microscope as quickly as possible, and begin recording. In our experience, images should be taken at inter-

vals not >15 s. Longer intervals will leave significant gaps in your data during rapid mitotic events, such as anaphase. Lengthy time averaged image collections should be avoided, because the rapid cortical motions result in blurred images. (See **Notes 3** and **4**).

2. Animation of your data will greatly facilitate its interpretation. The details of this process will vary depending on the equipment used to obtain the images. Our images are obtained with a Bio-Rad MRC-600 confocal system under the control of CoMOS software. We convert the CoMOS image files to TIFF format using Confocal Assistant, which runs under Windows. The TIFFs are transferred to a Macintosh computer and converted to a single PICS (PICT series) file using Graphic Converter (shareware, available at <ftp://mirror.archive.umich.edu/systems/info-mac/gst/grf/>). PICS files are converted to Quicktime movies with Adobe Premiere.

4. Notes

1. In order to limit the introduction of artifact, it is quite important that the embryos do not overheat and that they receive ample oxygen. We keep our confocal analysis room at a reduced temperature, which guards against overheating and reduces oxygen demand. This has the added advantage of slowing down the mitotic cycles, which means that a higher effective temporal resolution may be achieved. Covering your embryos with a minimal amount of halocarbon oil will further reduce the possibility of their developing hypoxia.
2. Although the majority of our experiments have employed 300-rad doses, greater or lesser amounts can be used depending on the desired effect. Since the lethal dosage needed to kill 50% of 1.5 h-old embryos is 180 rad, we consider this the minimum useful dosage (**4**).
3. When using rhodamine, one may collect images at short intervals without worrying about photobleaching, because rhodamine is a highly photo stable dye. We have been able to collect three-pass, Kalman averaged images at 10-s intervals for as much as an hour without appreciable loss of image quality.
4. It has been our experience that the GFP signal is quite resistant to photobleaching in control embryos, but may bleach fairly rapidly following X-irradiation. We believe that this may be owing to X-ray destruction of maternally contributed RNA and a subsequent decrease in replacement of bleached molecules by protein synthesis.

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Inhibition of DNA Synthesis by Ionizing Radiation

Nicolaas G. J. Jaspers and Malgorzata Z. Zdzienicka

1. Introduction

In mammalian cells, the rate of DNA synthesis decreases after X-ray exposure. The dose–response curve indicates a biphasic kinetics of inhibition (*see Fig. 1*). The initial, steep component of the curve represents inhibition of initiation of new replicons, whereas the shallow component is a manifestation of chain growth failure. The latter is probably owing to direct interference by DNA lesions with the replication machinery; on the other hand, slowdown of replicon initiation is one of the manifestations of an active signal-mediated response. Evidence for this was obtained from studies on cells derived from patients with the autosomal-recessive human diseases ataxia telangiectasia (AT) and Nijmegen Breakage Syndrome (NBS), and on some laboratory-generated radiosensitive rodent cell lines (*1,2*). AT and NBS cells have virtually lost the steep component of the inhibition curve. This property is usually referred to as “radioresistant DNA synthesis” (RDS), a phrase originally coined by Painter (*3*). In the rodent mutants, diminished replicon initiation is also evident, but the degree of DNA synthesis inhibition is generally lower in the parental hamster cell lines as well (*4,5*).

Although a number of biological end points after ionizing radiation exposure are abnormal in AT/NBS cells (*6*), DNA synthesis rate measurement is one of the more frequent assays used for biochemical support of the AT/NBS diagnosis. In no other inherited disorders with mutagen sensitivity or chromosomal instability, including those with moderate radiosensitivity (e.g., Huntington’s disease, inherited retinoblastoma, Cockayne syndrome), has the RDS phenotype been detected (*see Table 1*). This indicates that RDS is a typical feature of AT and NBS cells. To date, over 200 AT and NBS patients have

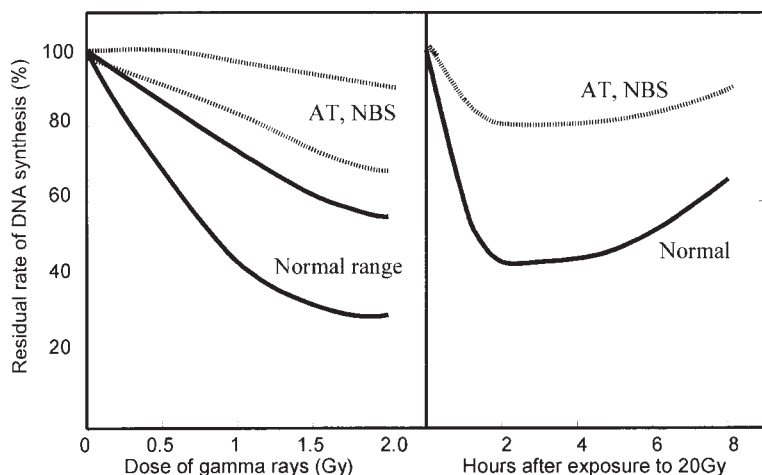


Fig. 1. Inhibition of DNA replication by ionizing radiation in primary human fibroblasts. Left, dose-response curve measured in the first 3 h after exposure. Right, time-course after exposure to 20 Gy of γ -rays. (Measured with 30-min labeling pulses).

been tested in this respect with almost invariably similar results (*see Fig. 1*). In very rare cases, DNA replication inhibition at intermediate or normal levels has been found. However, these cases always showed a nonstandard (mostly milder) clinical course of the disease, which was related either to a leaky mutation in the *ATM* gene (7–9) or to a different genetic entity resembling AT in some respect. Moreover, AT and NBS heterozygotes are claimed to be slightly radiosensitive, and cannot be distinguished from normal individuals with the RDS assay. Although direct screening of the large *ATM* gene for the wide range of mutations found in AT families is (still) impractical, DNA synthesis measurement will remain the assay of choice for diagnostic support in most cases (*see Note 1*).

Genetic heterogeneity in AT and NBS has been suggested on the basis of complementation of the RDS phenotype in somatic cell hybrids (10,11). However, the same *ATM* gene, on chromosome 11q22-23, was found to be mutated in all of the established AT complementation groups (7). Based on recent linkage data, all NBS patients, too, appear to be affected at a single *NBS1* gene, on 8q21 (12–15). The complexity of the mechanisms controlling the rate of DNA replication has been indicated by the existence of several genes responsible for inhibition of DNA synthesis after irradiation (2,16,17), in addition to *ATM* and *TP53* (18). In genetic analyses of rodent cell mutants, dominant-negative RDS patterns have been observed (16). Most probably, RDS is too distant from the primary defects in all these mutants, so that additional factors interfere with

Table 1
Patients Tested for RDS

RDS found	No RDS found
AT (<i>n</i> > 200)	Xeroderma pigmentosum (complementation groups A-G, XP variant) Cockayne syndrome (AB)
NBS (<i>n</i> > 25)	Trichothiodystrophy (XP-BD, TTDA, and UV ^R) Fanconi anemia (A-C) Bloom syndrome Dyskeratosis congenita Hutchinson-Gilford progeria Werner syndrome Teebi syndrome Dubowycz syndrome Retinoblastoma Li-Fraumeni syndrome Usher syndrome (type III) Synch syndrome (type II, HNPCC) Muir-Torre syndrome Familial adenomatous polyposis DNA ligase I deficiency Friedreich ataxia Porokeratosis mibelli Familial melanoma (p16) Down syndrome (and others)

regular complementation patterns. All these results indicate clearly that the biological end point of RDS cannot be used as a parameter in complementation studies.

Traditionally, overall DNA replication is best measured by incorporation of labeled thymidine, followed by some quantification method, such as liquid scintillation counting or autoradiography. The fact that the results of both techniques are very similar (10), one reflecting whole-cell cultures and the other single cells, indicates that over the labeling period, cell-cycle effects (e.g., efficiency of S-phase entry) do not play a major role. What is measured here is clearly a checkpoint governing S-phase-dependent DNA synthesis itself. Alternative DNA precursors, such as bromodeoxyuridine, have also come into use after sensitive immunofluorimetric detection methods became available.

This chapter describes the most straightforward and reproducible experimental protocol, making use of double-labeling. This procedure is highly

accurate and reproducible to within a few percent, and therefore also useful for clinical purposes.

2. Materials

1. Use a thymidine-free cell-culture medium formulation with moderate buffering capacity, such as RPMI 1640 or thymidine-free Ham's F10 (Gibco) (*see Notes 2 and 3*).
2. [^{14}C]-Thymidine, SA 0.05 Ci/mmol.
3. [Methyl- ^3H]-thymidine, SA from 2–50 Ci/mmol (or higher).
4. Phosphate-buffered saline (PBS).
5. 1 mM Thymidine in PBS.
6. 1 M Na-HEPES, pH 7.35. Filter-sterilize and store at room temperature.
7. 0.25 M NaOH, freshly prepared.
8. Scintillation cocktail suitable for alkaline solutions (e.g., Hionic Fluor, Packard).
9. 30-mm Cell-culture dishes.

3. Methods

3.1. Cell Prelabeling (*see Note 4*)

1. Seed cells into 30 mm dishes. **Make sure the cells are well dispersed.** Use $5\text{--}7 \times 10^4$ cells/dish. For transformed HeLa or hamster cells (e.g., CHO), use 1×10^4 cells/dish. Set up two dishes of cells to be irradiated and four dishes to be used as unirradiated controls. These six dishes will give a single point on a dose–response curve. Culture for 20–30 h.
2. Incubate the cells overnight (15–17 h) in the presence of [^{14}C]-thymidine, 0.05 $\mu\text{Ci/mL}$ (molar concentration = 1 μM). Add HEPES buffer to a final concentration of 20 mM.

3.2. Radiation Exposure (*see Notes 5 and 6*)

Take the cells to the radiation source, preferably without changing the ^{14}C -medium. If a change of medium is required for safety reasons, use standard medium at room temperature with additional HEPES for strict pH control (*see Note 4*). Cooling down to below 5°C is undesirable (the cold shock compromises the control of DNA synthesis) and unnecessary, since repair of most of the single- and double-strand breaks is fast (within a few minutes and 30 min, respectively) in comparison to the labeling period. With most ionizing radiation sources, dose rates are on the order of 1–2 Gy/min.

3.3. Second Labeling

1. Remove the ^{14}C -medium and immediately add tritiated HEPES-buffered medium. Use [methyl- ^3H]-thymidine at 2.0 $\mu\text{Ci/mL}$. With higher specific activities, add unlabeled thymidine to a final molar concentration of 1 mM to reduce the effec-

- tive specific activity and produce a predictable $^3\text{H}/^{14}\text{C}$ ratio of around 10 (*see Subheading 3.5. and Note 7*).
2. Incubate for 3–4 h.

3.4. Collection of Labeled Cells

Any measurement of incorporation requires the removal of free labeled precursors from the cells. The classical way of doing this is by acid (trichloroacetic acid, TCA) precipitation (*see Note 8*). However, the following protocol is a greatly simplified alternative procedure:

1. Remove the medium, and rinse the cells with warm (37°C) PBS.
2. Incubate at 37°C with unlabeled medium for 20–30 min, to chase the labeled precursor pool.
3. Remove the medium and rinse the cells once with PBS.
4. Squirt 500 μL of fresh 0.25 *M* NaOH over the monolayer to lyse the cells.
5. Pipet-mix the lysate, and transfer to a scintillation vial.
6. Mix the lysate well with 7.5 mL of scintillation cocktail. The solution should be clear.

3.5. Scintillation Counting

Make use of a dual-label counting program with in-line standard quenching curves, that discriminates between ^3H and ^{14}C and calculates the corrected disintegration/minute (dpm) values for you. Checking proper action of this program with standard amounts of labeled mixtures is strongly advised. Counting time/vial should be 5 min at least. The dpm calculations are only accurate within certain limits: $^3\text{H}/^{14}\text{C}$ dpm ratios should always be within 2.0 and 15.0 for all irradiated samples. Values outside this range should be seriously mistrusted because of this calculation problem and for other reasons (*see Note 7*).

3.6. Processing Data into Inhibition Curves

Use the $^3\text{H}/^{14}\text{C}$ ratios as a measure for the overall rate of DNA synthesis. Duplicate values are usually within 5%. Any spreadsheet program is suitable to process the data into standard inhibition curves. If standard errors (SE) are calculated and presented in the curves, the SE obtained in the unexposed controls should be transported into the data points. (*See Note 9*.)

4. Notes

1. Clinical applications: The RDS test is the routine assay for prenatal diagnosis of AT and NBS (*19,20*), although the use of polymorphic genetic markers located near the *ATM* gene has become an attractive alternative (*9*). Whereas the latter has the significant advantage of direct processing of chorionic samples without culture, it is by no means simple and still requires highly experienced hands, blood sampling of family members, and a range of experimental controls. Of

course, the molecular genetic tool can only be considered after firm establishment of the diagnosis in the index patient, which will frequently entail a cellular radiation exposure test (such as DNA synthesis inhibition), too.

2. Do not use DMEM. It is not possible to keep the pH stable within the narrow range required, even with added HEPES. This will lead to experimental variability. (DMEM is buffered well in the presence of 10% CO₂ in the incubator, but removing it from the incubator causes problems with pH control.) Media such as RPMI 1640 and Ham's F10 and F12 maintain a stable pH. If using Ham's F10, it is necessary to order a special thymidine-free batch.
3. Dialysis of the serum supplement to remove unlabeled thymidine (otherwise present at 0.1–0.15 μ M) is not required with the labeling protocol used here, and only marginally increases overall label uptake.
4. A DNA synthesis experiment is not at all complicated. However many investigators have reported difficulties with reproducibility. To obtain reproducible results:
 - a. Minimize handling and temperature changes.
 - b. Add HEPES buffer for strict pH control during all labeling periods.
 - c. Include a prelabeling to have an internal standard, facilitating experimental handling.

The first and second points are particularly vital, since the overall rate of DNA replication in cultures responds immediately to sudden changes in many physical parameters, including temperature, pH, and osmotic pressure, even if they are small.

5. Radiomimetic chemical agents: Incubation with other agents, such as bleomycin or adriamycin (usually for 30–60 min), should always be performed immediately after prelabeling, and in the presence of supplementary HEPES, to strictly control the pH.
6. In primary human fibroblasts irradiated with <10–15 Gy, inhibition largely derives from changes in the fast component of the curve. This dose range is therefore sufficient to study inhibitory effects on replicon initiation, which largely reflects signal-based control of DNA replication rate. Above 10–20 Gy, the minimal rate of overall replication is reached between 1.5 and 3 h. This implies that a good picture of DNA synthesis inhibition is best obtained in a pulse-labeling protocol of 2–4 h. Shorter times tend to produce more technical variation, without much gain in information.
7. Very low ³H/¹⁴C ratios may indicate that the dishes were too full, and the cells have started to reach confluence. In this case, discard the experiment. Very high ratios indicate serious problems in the initial phase of the experiment or point to microbial contamination (e.g., mycoplasma). The ¹⁴C dpm count/dish should be 2–7 $\times 10^3$ with primary fibroblasts; 10-fold higher values are usual with transformed cells. Incorporation levels of <1000 dpm/dish are unacceptably low: discard such an experiment.
8. Alternative protocol: TCA precipitation.
 - a. Remove the medium, and rinse the cells with ice-cold PBS + 1 mM “cold” thymidine.

- b. Scrape the cells with a rubber policeman into 1 mL of ice-cold PBS.
 - c. Pipet the cells onto a glass filter disk soaked in 10% TCA (w/v), and apply gentle vacuum suction.
 - d. Rinse the filter once with ice-cold 10% TCA and twice with ice-cold ethanol.
 - e. Put the filter in a scintillation vial, and dry in an oven. Add 0.5 mL of solubilizer, e.g., Soluene.
 - f. After 15–30 min, add scintillation fluid, mix well, and count.
9. Strain variability: The degree of inhibition of DNA replication by ionizing radiation is widely different among cell lines, with human cells showing, in general, much more pronounced inhibition of DNA synthesis than rodent cells, such as Chinese hamster V79 and CHO9 cells, although mouse A9 cells respond similarly to human cells (4,5,16). The hamster cell mutants defective in Ku86 show a more pronounced inhibition than their parental cell lines (4). Since transformed and tumor cells are characterized by seriously compromised growth control, diverse responses are to be expected in the steep component of the inhibition curve, which is in fact what is found. In addition, commonly used transformed strains may have very different average replicon sizes, which affect the shallow component of the inhibition curve.

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Analysis of Inhibition of DNA Replication in Irradiated Cells Using the SV40-Based In Vitro Assay of DNA Replication

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1. Introduction

Inhibition of DNA replication in eukaryotic cells was one of the earliest effects of radiation to be reported and quantitated. The elucidation of the mechanism of this inhibition has been the focus of research in several laboratories for four decades. A significant development in recent years is the recognition that the mechanism of this inhibition has both direct (*cis*-acting) and indirect (*trans*-acting) components (for a review, *see I*). Whereas the direct component is thought to derive from radiation-induced DNA damage that alters chromatin structure and inhibits DNA replication in *cis*, the indirect component is attributed to the activation by DNA damage of regulatory processes that inhibit DNA replication in *trans*. The latter mechanism of inhibition is equivalent to the activation of a checkpoint in S-phase (2–5).

The recognition that a checkpoint is activated in S-phase after induction of DNA damage has led to intensive studies of its genetic and biochemical basis. The best-documented genetic alteration that affects the regulation of DNA replication in response to radiation exposure is found in individuals with the hereditary genetic disorder ataxia telangiectasia (AT). AT cells fail to inhibit DNA replication in response to DNA damage (*see* Chapter 44), suggesting that *ATM*, the gene mutated in these cells (6), is normally involved in the regulation of DNA replication (7,8). Other components of the signal transduction cascade that transmits signals to the DNA replication machinery may include growth factor receptors, genes of the *ras* and *myc* families, as well as of yet unidentified protein kinases. Finally, replication protein A (RPA) has been implicated

in the downregulation of DNA replication following exposure to DNA damaging agents (reviewed in *1*).

The operation of a checkpoint during S-phase has recently been confirmed by genetic studies in the budding yeast, and genes responsible for this response have been identified (*9,10*). Genes involved in the regulation of DNA replication after DNA damage are expected to act either as DNA damage sensors or modifiers, or to be members of the signaling pathway that communicates with the replication machinery (*2*). Elucidation of the functions of known genes and the discovery of new genes of the regulatory pathway are major goals of current research in the topic.

Promising for the delineation of the mechanism of the S-phase checkpoint is the observation that factors that inhibit DNA replication *in vivo* can be found in active form in extracts prepared from irradiated cells when these are tested for replication activity using the simian virus 40 (SV40) assay for *in vitro* DNA replication (*11–13*). In this assay, replication of plasmids carrying the minimal origin of SV40 DNA replication is achieved *in vitro* using cytoplasmic cell extracts and TAg as the only noncellular protein (*14–17*). It is thought that cellular proteins function in this assay in the same manner as *in vivo*. The assay has been extremely successful in the field of DNA replication and has led to the characterization of a number of factors involved in eukaryotic DNA replication. It is thought that in a similar way the assay will allow the biochemical characterization of important components of the regulatory pathway activated in response to DNA damage.

Here, we describe protocols developed to measure *in vitro* DNA replication with the purpose of analyzing its regulation after exposure to DNA damage. The required procedures include:

1. Preparation of cytoplasmic extract from cells that have sustained DNA damage.
2. Preparation of the SV40 large tumor antigen (TAg).
3. Preparation of supercoiled plasmid DNA carrying the SV40 origin of DNA replication.
4. Assembly of *in vitro* replication reactions.
5. Assay of DNA replication activity using incorporation of radioactive precursors, and of the DNA replication products using gel electrophoresis.

Figure 1 shows a graphic outline of these steps.

2. Materials

2.1. Preparation of HeLa-Cell Extract

1. Minimum Essential Medium (MEM) modified for suspension cultures (S-MEM), supplemented with 5% iron-supplemented bovine serum and antibiotics (penicillin 100 U/mL, streptomycin 100 µg/mL).

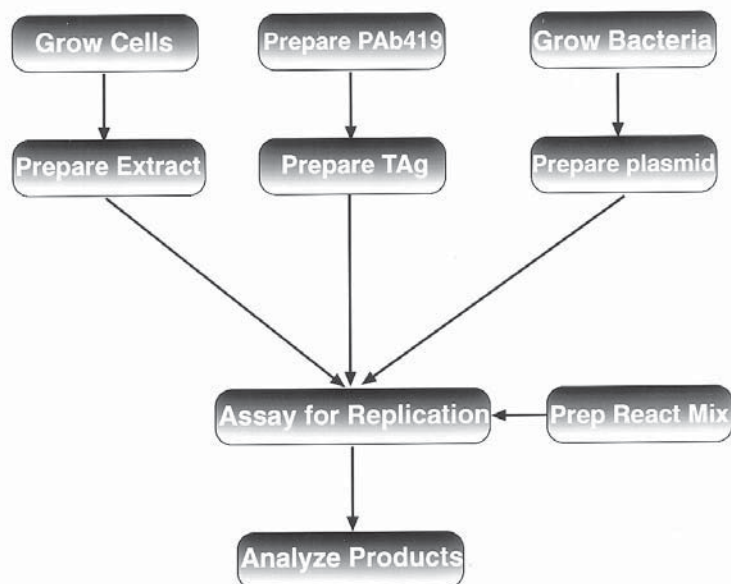


Fig. 1. Outline of the individual steps and preparations required for assembling DNA replication reactions using the SV40 system for *in vitro* DNA replication and for evaluating their outcome.

2. Hypotonic buffer solution: 10 mM HEPES, pH 7.5 (stock 0.6 M, pH 7.5 at room temperature), 1.5 mM MgCl₂ (stock 1 M), 5 mM KCl (stock 3 M). Immediately before use, add 0.2 mM phenylmethylsulfonyl fluoride (PMSF) (stock 100 mM in isopropanol), and 0.5 mM dithiothreitol (DTT) (stock 1 M in H₂O; store at -20°C), 20 mM β-glycerophosphate.
3. 10X cytoplasmic buffer: 10 mM HEPES, pH 7.5, 1.4 M KCl, and 1.5 mM MgCl₂.
4. Dialysis buffer: 25 mM Tris-HCl, pH 7.5 at 4°C (stock 1 M, pH 7.5 at 4°C), 10% glycerol, 50 mM NaCl (stock 5 M), 1 mM EDTA (stock 0.5 M, pH 8.0). Immediately before use add 0.2 mM PMSF, 0.5 mM DTT, and 20 mM β-glycerophosphate.
5. Microcarrier spinner flasks of 30-L nominal volume; Bellco Glass Inc (Vineland, NJ).
6. Microcarrier magnetic stirrers; Bellco Glass Inc.
7. Tissue-culture dishes (100 mm).
8. Dounce homogenizer with B pestle, 50 mL.

2.2. Preparation of TAG

Investigators can either obtain this protein from a commercial source (CHIMERx, Milwaukee, WI) or prepare it in the laboratory using available reagents as follows:

1. Hybridoma cell line PAb419. This is the L19 clone generated by Harlow et al. (18). It produces a monoclonal antibody (MAb) that recognizes the amino-terminal

region of TAG and is used for the preparation of immunoaffinity columns employed in the purification of TAG. The cell line can be requested from E. Harlow, and is used to produce antibody that can be purified using standard procedures (19).

2. Insect cells (*Sf9*): Available from ATCC (Manassas, VA).
3. Baculovirus *Autographa californica* expressing TAG: This virus was constructed by Lanford (941T) (20) using a cDNA copy of TAG mRNA and can be requested from the author. The procedures used to prepare stocks of the virus and to measure its infectivity in pfu/mL are described in specialized protocols, and the reader is referred to these publications for more information, e.g., (21).
4. TD buffer: 25 mM Tris-HCl (stock 1 M, pH 7.4), 136 mM NaCl (stock 5 M), 5.7 mM KCl (stock 3 M), 0.7 mM Na₂HPO₄ (stock 0.2 M).
5. Buffer B: 50 mM Tris-HCl (stock 1 M, pH 8.0), 150 mM NaCl (stock 5 M), 1 mM EDTA (stock 0.5 M, pH 8.0), 10% glycerol, 1 mM PMSF (stock 0.1 M), 1 mM DTT (stock 1 M).
6. Buffer C: 50 mM Tris-HCl (stock 1 M, pH 8.0), 500 mM LiCl (stock 1 M), 1 mM EDTA (stock 0.5 M, pH 8.0), 10% glycerol, 1 mM PMSF (stock 0.1 M), 1 mM DTT (stock 1 M).
7. Buffer D: 10 mM PIPES (stock 1 M, pH 7.4, dissolved in 1 M NaOH), 5 mM NaCl (stock 5 M), 1 mM EDTA (stock 0.5 M, pH 8.0), 10% glycerol, 1 mM PMSF (stock 0.1 M), 1 mM DTT (stock 1 M).
8. Buffer E: 20 mM triethylamine, 10% glycerol, pH 10.8. Make just before using.
9. Buffer F: 10 mM PIPES (stock 1 M, pH 7.0), 5 mM NaCl (stock 5 M), 0.1 mM EDTA (stock 0.5 M, pH 8.0), 10% glycerol, 1 mM PMSF (stock 0.1 M), 1 mM DTT (stock 1 M).
10. 0.2 M Sodium borate, pH 9.0.
11. Dimethylpipelimidate.
12. 0.2 M Ethanolamine, pH 8.0.
13. Merthiolate.
14. 10% Nonidet P-40 (NP-40).
15. Chromatography supplies: protein A agarose, Sepharose 4B-Cl (Pharmacia, Piscataway, NJ), Syringes (5 mL), or EconoColumns with id of 0.75 cm (Bio-Rad, Melville, NY).
16. Supplies and equipment for SDS-PAGE.
17. Two 1-L and one 250-mL microcarrier spinner flasks (Bellco).
18. Material for protein determination using the Bradford assay (e.g., Protein Assay kit, Bio-Rad).

2.3. Preparation of Supercoiled Plasmid DNA Carrying the SV40 Origin of DNA Replication

Several plasmids that carry the minimum origin of SV40 DNA replication are available and can be used for this purpose e.g., pSV01ΔEP (22), pSV010 (23), and pJLO (24). Large quantities of these plasmids can be prepared using cesium chloride/ethidium bromide gradients. Details of these procedures can be found in Chapter 8 or in publications describing molecular biology protocols (21,25).

2.4. Assembly of *In Vitro* Replication Reactions and Product Analysis

1. Replication reaction solution (5X): 200 mM HEPES-KOH, pH 7.5, 40 mM MgCl_2 , 2.5 mM DTT, 200 mM Phosphocreatine, 15 mM ATP, 1 mM CTP, 1 mM GTP, 1 mM UTP, 0.5 mM dATP, 0.125 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP. Prepare 10-mL replication reaction solution, and freeze in small aliquots.
2. Creatine phosphokinase stock: 2.5 mg/mL prepared in 50% v/v glycerol. Phosphokinase together with phosphocreatine present in the replication reaction solution form an ATP regeneration system that is required for DNA replication.
3. Salmon sperm DNA.
4. 0.5 M EDTA, pH 8.0.
5. 10% Trichloroacetic acid (TCA).
6. 10% Sodium dodecyl sulfate (SDS).
7. Proteinase K.
8. RNase A.
9. Scintillation Counter.
10. Gel electrophoresis equipment.
11. Glass fiber filters (GF/C, Whatman, Clifton, NJ).

3. Methods

3.1. Preparation of Cell Extract (see Notes 1 and 2)

The method described here allows the preparation of cell extract from 10 L of cell suspension. Higher or lower amounts of extract can be prepared by appropriate scaling. Ionizing radiation or radiomimetic chemicals can be used for the generation of damage in the DNA. We obtained satisfactory results using a 3-h treatment with 0.5 $\mu\text{g/mL}$ camptothecin, a DNA topoisomerase I inhibitor. We also use routinely 10–40 Gy of 25 MV X-rays from a linear accelerator. The details for the latter treatment depend heavily on the type of equipment used and is therefore impractical to describe them here. Investigators with access to such equipment are advised to request the assistance of radiation physicists for dosimetry and other details on the irradiation protocol. Standard X-ray-producing equipment (50–250 kV) cannot be used to irradiate volumes of the magnitude described here owing to their low penetration characteristics. Whatever the solution for the irradiation problem, it is important to keep in mind that disturbance in the cell culture has to be kept to a minimum before and after irradiation for reproducible results. Significant reductions in temperature, changes of the growth vessels, centrifugations, and so forth, should be avoided. Extracts can be processed for repair at a specific time after irradiation. The precise timing will depend on the type of experiment and its specific goal. We prepare extracts 0–3 h after DNA damage induction.

1. Grow HeLa cells at 37°C for 3 d in 25 100-mm tissue-culture dishes prepared at an initial density of 6×10^6 cells/dish in 20 mL of S-MEM supplemented with

serum and antibiotics. The final density after three days of growth should be $\sim 20 \times 10^6$ cells/dish, giving a total of 5×10^8 cells in 25 dishes.

2. Trypsinize cells from all the dishes and resuspend in 10 L of prewarmed complete growth medium in a 30-L nominal-volume microcarrier flask, thoroughly pregassed with 5% CO₂ in air. The initial cell concentration should be $\sim 5 \times 10^4$ cells/mL. Place in a warm room at 37°C; provide adequate stirring (~ 40 – 60 rpm).
3. Allow the cells to grow for 4 d, to a final concentration of 4 – 6×10^5 cells/mL. Do not exceed this concentration of cells.
4. Collect the cells by centrifugation (8 min at 2500g). Collection should be fast and is best done using a refrigerated centrifuge that can accept 1-L bottles (e.g., Beckman J6-MI, Fullerton, CA). All further processing should be carried out at 0–4°C.
5. Rinse twice in PBS, and centrifuge (5 min at 500 g). Determine the packed cell volume (PCV) (~ 12 – 15 mL total).
6. Resuspend the cell pellet in 5 PCV of hypotonic buffer solution, and centrifuge quickly (5 min, 500g). Cells swell, and the PCV approximately doubles.
7. Determine the new PCV. Resuspend the cell pellet in 3 PCV of hypotonic buffer, and disrupt in a Dounce homogenizer (20 strokes, pestle B). It is advisable to test cell disruption using a phase-contrast microscope.
8. Add 0.11 vol of high-salt buffer, and centrifuge at 3000g for 20 min.
9. Carefully remove the supernatant and centrifuge at 100,000g for 1 h.
10. Place the resulting extract (S100) in dialysis tubing with a mol-wt cutoff of 10–14 kDa, and dialyze overnight against 50–100 vol of dialysis buffer.
11. Collect the extract. Centrifuge at 15,000g for 20 min to remove precipitated protein. Aliquot and snap-freeze. Store at -70°C . Keep a small aliquot for determining protein concentration using the Bradford assay.

3.2. Preparation of TAG

Good quality TAG is essential for efficient *in vitro* replication of plasmids carrying the SV40 origin of DNA replication. The method of preparation described here is essentially the one described by Simanis and Lane (26), and can be conveniently separated into two parts:

1. Preparation of the TAG immunoaffinity column.
2. Purification of TAG from extracts of *sf9* cells infected with the baculovirus 941T, which expresses TAG.

3.2.1. Preparation of TAG Immunoaffinity Column

1. Mix 5 mg of PAB419 antibody with 2 mL of wet protein A beads. Incubate at room temperature for 1 h with gentle rocking.
2. Wash the beads twice with 20 mL of 0.2 M sodium borate (pH 9.0) by centrifugation at 1000g for 5 min.
3. Resuspend the beads in 20 mL of 0.2 M sodium borate (pH 9.0), mix, and remove 100 μL of bead suspension for assaying coupling efficiency. Add solid dimethylpipelimitate to bring the final concentration to 20 mM.

4. Mix on a rocker for 30 min at room temperature, and remove a 100- μ L suspension of the coupled beads.
5. Stop the coupling reaction by washing the beads with 20 mL of 0.2 *M* ethanolamine (pH 8.0), and incubate for 2 h at room temperature in 0.2 *M* ethanolamine with gentle rocking.
6. Spin down and resuspend the beads in PBS. Add 0.01% merthiolate if extensive storage is anticipated. At this point, the beads are ready for use in the purification of TAG (if the quality-control performed next is positive).
7. Check the efficiency of coupling by boiling in Laemmli sample buffer the samples of beads taken before and after coupling. Run the equivalent of 1 and 9 μ L of beads from both samples on a 10% SDS-PAGE gel and stain with Coomassie blue. Good coupling is indicated by heavy chain bands (55 kDa) in the samples obtained before, but not in the samples obtained after coupling.
8. Prepare the immunoaffinity column by pouring beads into a 5-mL syringe, or a 0.75-cm diameter EconoColumn.

3.2.2. Purification of TAG from Extracts of *sf9* Cells

1. Grow enough *sf9* cells to prepare 1 L of cell suspension at 2×10^5 cells/mL. Distribute the cell suspension in two 1-L microcarrier spinner flasks (500 mL of cell suspension/spinner) and incubate at 27°C under gentle spinning (1–1.5 revolutions/s). Allow the cells to grow until they reach a concentration of 2×10^6 cells/mL (3–4 d). (See **Note 3**.)
2. Spin cells for 5 min at 500g, and carefully return the supernatant to the spinner flasks.
3. Resuspend the cells in enough volume of virus stock to reach a multiplicity of infection equal to 10 *pfu*/cell. Place the cell suspension in a 250-mL spinner flask, and allow attachment of virus to the cells by gentle stirring at 27°C for 2 h.
4. Return the cell suspension to the original spinner flasks, and incubate for 48 h at 27°C under gentle stirring to allow for protein expression.
5. Harvest the cells by centrifuging at 500g for 5 min, and resuspend in 25 mL of TD buffer.
6. Centrifuge at 500g for 5 min, and resuspend in 30 mL of buffer B. Add 10% NP-40 to a final concentration of 0.5%.
7. Place in ice for 30 min. Invert the tube several times every 10 min.
8. Centrifuge for 10 min at $\sim 10,000g$ in a corex tube. At this stage, the extract can be removed and quickly frozen at -70°C for use at a later time to purify TAG. When needed, this extract is thawed quickly by immersing in warm water. Do not allow the extract to warm up above 4°C. All subsequent steps should be carried out in a cold room.
9. Save a 50- μ L aliquot of the cell extract, and load the remaining material onto a 2-mL 4B-CI Sepharose column equilibrated with buffer B. Elution can be achieved either by gravity, or with the help of a peristaltic pump giving a flow rate of approx 1 mL/min. This step retains proteins binding nonspecifically to agarose.

10. Allow the flowthrough of **step 9** to pass through a 2-mL protein A Sepharose column equilibrated with buffer B. This can be achieved either by gravity or with the help of a peristaltic pump giving a flow rate of approx 1 mL/min. This step retains material binding nonspecifically to protein A.
11. Allow the flowthrough of **step 10** to pass through a 2-mL immunoaffinity column of protein A agarose coupled with antibody PAb419. This can be achieved either by gravity or with the help of a peristaltic pump at a flow rate of approx 0.5 mL/min. This step retains TAg from the cell extract.
12. Repeat **step 11**. Save the flowthrough.
13. Wash the immunoaffinity column with 200 mL of buffer C. This is best achieved with a peristaltic pump giving a flow rate of approx 2 mL/min.
14. Wash the immunoaffinity column with 100 mL of buffer D. This is best achieved with a peristaltic pump giving a flow rate of approx 2 mL/min.
15. Elute with buffer E (*see Note 4*). Collect 0.5-mL fractions in tubes containing 25 μ L of 1.0 M PIPES, pH 7.0. Place the fractions in ice.
16. Wash the immunoaffinity column with 20 mL of buffer E, and then with 40 mL of buffer B. The column can be reused four to five times.
17. Measure the protein concentration using the Bradford assay.
18. Combine fractions containing protein. Dialyze overnight against 2 L of buffer F.
19. Test the purity by SDS-PAGE followed by silver staining. Test the activity for in vitro SV40 DNA replication. The procedure yields 1–2 mg TAg/L of *sf9* cell culture.

3.3. Preparation of Supercoiled Plasmid DNA Carrying the SV40 Origin of DNA Replication

Standard procedures can be used for the preparation of supercoiled plasmid DNA. It is preferable to purify the DNA using a two-step purification on cesium chloride/ethidium bromide gradient. Detailed protocols for this purpose can be found in Chapter 8 or in other sources of protocols (21,25).

3.4. Assembly of In Vitro Replication Reactions and Evaluation of Replication Activity

1. Assemble 50- μ L reactions by mixing in an Eppendorf tube kept in ice, 5 μ L of reaction buffer, 2.5 μ L of creatine phosphate, 200–400 μ g of extract protein, 1 μ g of TAg, 0.3 μ g of superhelical plasmid DNA, and 0.001 μ Ci/mL of (α - ^{32} P)dCTP; add H₂O to 50 μ L. Extracts from untreated and treated cells should be used in parallel, so that the results obtained can be directly compared.
2. Incubate the reactions at 37°C for 1 h. Longer or shorter incubations can also be used if information on the kinetics of replication is desired.
3. Terminate the reactions by adding EDTA to a final concentration of 20 mM.
4. Add 25 μ g of denatured salmon sperm DNA, and mix well.
5. Add 1 mL of cold 10% TCA to precipitate acid-insoluble material. Mix well.
6. Collect the precipitate onto Whatman GF-C glass fiber filters. Wash three times with 10 mL of cold 10% TCA. Wash four times with 10 mL of deionized water.

7. Add 5 mL of scintillation fluid. Measure incorporated activity in a scintillation counter.

3.5. Analysis of the Replication Products

1. To analyze the DNA replication products by gel electrophoresis, add to the samples after completion of replication SDS to 0.1%.
2. Digest with RNase (20 $\mu\text{g/mL}$) for 15 min at 37°C.
3. Add proteinase K (200 $\mu\text{g/mL}$), and incubate at 37°C for 30 min.
4. Purify the DNA either by extraction in phenol/chloroform followed by precipitation in ethanol or by using commercially available DNA purification systems.
5. Electrophorese in 1% agarose at 6.5 V/cm for 2 h. Electrophoretic conditions may need to be modified if larger plasmids are used. For optimal resolution, reduce the field intensity to 1 V/cm.

4. Notes

1. The preparation of a good extract depends strongly on the quality of the cells used. When cell growth is not optimal, or when cells overgrow, low replication activity may be obtained, and the inhibition in extracts of treated cells may be suboptimal. To ensure optimal growth, we routinely take the following measures:
 - a. Carefully test different batches of serum to find one with good growth characteristics. HeLa cells have a generation time of <20 h when grown as a monolayer and <24 h when grown in suspension, under optimal growth conditions.
 - b. Use cells grown in dishes to start the suspension cultures for extract preparation. This helps to reduce clumping after extensive growth in suspension.
 - c. We follow cell growth daily, and collect cells for extract preparation when they reach a concentration of $4\text{--}6 \times 10^5$ cells/mL.
 - d. We measure cell-cycle distribution by flow cytometry. A high percentage of S-phase cells (~25% for HeLa cells) suggests that the cell culture is still in an active state of growth.
2. The effect of the DNA damage-inducing agent on DNA replication *in vitro* can be variable. We found that this is usually owing to the growth conditions (overgrown cultures) or to the absence of phosphatase inhibitors. We routinely add β -glycerophosphate, since we found it to significantly improve the reproducibility. Other phosphatase inhibitors, as well as the use of protease inhibitors, should be considered if reproducibility problems persist.
3. Optimal cell growth is a prerequisite of a successful preparation of TAG. We find that *sf9* cells grow more consistently if kept in suspension. Transfer from a monolayer state to a suspension state is usually associated with a shock that takes the cells some time to overcome.
4. We have observed that 20 mM triethylamine may not elute all bound TAG from the immunoaffinity column. If this proves to be the case, increasing the triethylamine concentration (up to 100 mM), or alternative eluting methods (see **ref. 19**) should be considered. However, it should be kept in mind that such alternatives may reduce TAG activity.

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Assays of Bypass Replication of Genotoxic Lesions in Mammalian Disease and Mutant Cell-Free Extracts

Daniel L. Svoboda and Jean-Michel H. Vos

1. Introduction

1.1. *Aim*

The genotoxic consequences of DNA damage in living organisms include short-term genetic instability and programmed cell death, as well as long-term inheritance of mutations and somatically acquired cancer. To respond to such constant genotoxic insults, living creatures from viruses to humans have evolved the capacity to remove or tolerate DNA lesions. Although the first process is generally referred to as DNA repair, the latter one has been described in the eukaryotic literature as postreplication repair, translesion synthesis, or bypass replication. Numerous lines of evidence suggest that replication of DNA lesions is often an essential triggering factor in the induction of deleterious genetic effects (*1*). First, proliferating cells are more susceptible to neoplastic transformation than nonproliferating cells after genotoxic treatment. Second, mutation rates increase dramatically during S-phase of cells pre-exposed to DNA-damaging agents. Third, DNA damage stimulates replication-dependent clastogenic phenomena in mammalian cells, such as sister-chromatid exchanges, chromosomal aberrations, and gene amplification. Fourth, several cancer-prone syndromes present constitutional abnormalities in the recovery of replication after DNA damage (*see* Chapter 44). Because of such clear evidence for the primary role played by replication of DNA lesions on processes leading to genetic instability, it is important to understand the mechanisms of lesion persistence in eukaryotic cells.

One can safely predict that several mechanisms of replication of damaged chromosomes coexist in eukaryotic cells. Such redundancy is probably required

to fulfill distinct, complementary, or sometimes opposing roles necessary for the short-term and long-term survival of individual cells and complex multicellular organisms. Replication of lesion-containing DNA templates is widely documented in eukaryotes (1,2). First, in vivo experiments on the genome overall have clearly demonstrated the persistence of various genotoxic lesions, such as the prototypical UV-induced cyclobutane pyrimidine dimer, in replicated DNA of mammalian cells (3–6). Second, persistence of DNA damage has been directly demonstrated in newly-replicated, individual mammalian genes with the recent development of sensitive methods to detect and quantitate lesions at the single-copy level of mammalian genomes (7–10). Altogether, these studies with intact cells have firmly established that mammalian species can express effective mechanisms for replicating bulky genotoxic lesions in chromosomal DNA.

Paradoxically, despite the considerable evidence of bypass replication of DNA damage in mammalian cells, the mechanisms and fidelity of such lesion-tolerance processes are still poorly understood (Fig. 1) (1,2). Therefore, there is a fundamental need to develop strategies to isolate the genes and proteins involved in these pathways. One way to delineate a pathway is to develop an in vitro assay reproducing its main enzymatic reactions in a cell-free system; alternatively, one can identify cell mutants defective in this pathway and use them for cloning the corresponding genes. In recent years, both biochemical and genetic strategies have been followed in an effort to elucidate the process of bypass replication in higher eukaryotic cells. This chapter describes the methodologies currently used in our laboratories to reach this goal.

1.2. Strategy

1.2.1. *In Vitro SV40 Origin-Initiated Replication Is a Model for the Enzymology of Chromosomal Replication*

Several laboratories have developed an SV40-based system for studying human DNA synthesis in vitro (11–13). This powerful approach has resulted in the isolation of many factors involved in bidirectional, semiconservative DNA replication. Indeed, an in vitro system reconstituted from components purified to apparent homogeneity and exhibiting the mechanistic characteristics of unfractionated cell extract has been fully elucidated (14,15).

In the SV40-based system, the concerted action of at least 22 polypeptides is required to accomplish chromosomal DNA replication (15,16), followed by resolution (decatenation) and supercoiling of the daughter molecules (13). A virus-encoded protein, large T-antigen (TAg), is required to initiate SV40 origin activity (17). TAg binds to the origin sequence (18) and stimulates bidirectional template unwinding ahead of two oppositely directed replication forks

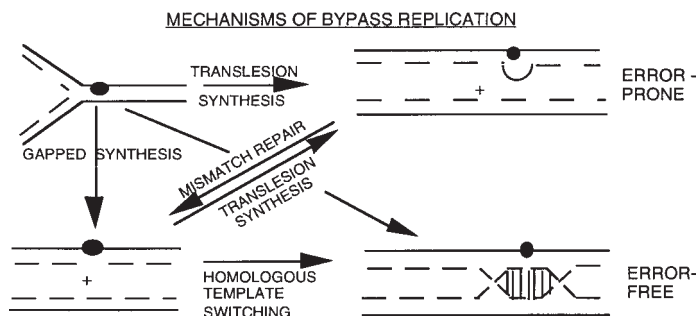


Fig. 1. Models for error-prone and error-free bypass replication of a bulky genotoxic lesion in human cells. The lesion is represented by the filled circle, and a mutation opposite the lesion is indicated as a half-circle. Continuous lines, parental strand; discontinuous lines, daughter strands.

(19). Replication protein A (RP-A), a single-stranded DNA binding protein, attaches to the unwound, single-stranded DNA, providing structural integrity for the replication fork (20). DNA polymerase α /primase binds to TAG (21–23), initiating RNA primer synthesis and initial elongation (24–27). A switch from the initiation polymerase to polymerase δ occurs through the recognition of free DNA 3'-ends by a complex of replication factor C (RF-C) and proliferating cell nuclear antigen (PCNA) (28–30). DNA polymerase δ then performs highly processive elongation of leading and lagging strands in conjunction with PCNA (28–30). Polymerase α /primase continues to perform lagging strand initiation as the replication fork progresses (31–33). Topoisomerase I manages superhelicity ahead of the replication fork (34). Maturation and ligation of Okazaki fragments ensues through the action of RNaseH/MF1 exonuclease and DNA ligase I (14,35–37). Topoisomerase II effects decatenation of daughter molecules (36). It should be stated that the purified system represents a minimal reconstitution. There is active debate over the actual identity of the elongation polymerase(s). DNA polymerase ϵ is essential for viability in yeast (38), and may be involved in addition to polymerase δ in leading and/or lagging strand elongation (39,40).

1.2.2. Detailed Mechanistic Study Using a Template with Specifically Placed Base Modifications

We have established a procedure that has yielded novel mechanistic and quantitative information on the replication of the *cis,syn*-thymine dimer (T<>T), the most frequent UV-induced DNA photoproduct (41). Site-specific placing of T<>T in either the leading or lagging strand of the replication fork has enabled, for the first time, unambiguous quantitation of rates of repli-

cation past T<>T in each strand. Replication rates for leading and lagging strand T<>T were found to be significantly different, prompting the hypothesis that different DNA polymerases are responsible for insertion of nucleotides at T<>T sites in the two strands. This result also pointed to the possibility that different mechanistic modes of nucleotide insertion may be operational here, e.g., direct (continuous) translesion synthesis at the replication fork vs (discontinuous) daughter strand gap filling. Therefore, by high-resolution mapping of label incorporation in sequences flanking T<>T in both strands of daughter molecules, we were able to provide evidence supporting the model proposed by Meneghini and Hanawalt in 1976 (42).

1.2.3. Defective T<>T Postreplication Repair in an Xeroderma Pigmentosum-Variant Cell Extract In Vitro

An immortal, rapidly proliferating, SV40 TAg-transformed XP-V cell line, designated CTAG, has been isolated (43). CTAG yields cell extract that is highly active in the SV40 origin-based in vitro replication assay (43–45). Using the in vitro SV40 replication assay and the T<>T-containing template, and by comparing CTAG extract with normal, we have obtained clear evidence of defective postreplication repair on a specifically placed T<>T-containing template in both leading (45) and lagging (Fig. 2; see refs. 41–45) strands. An Epstein-Barr virus (EBV)-transformed lymphoblastoid cell line from a different XP-V patient (GM02449B) gave similar results, providing additional evidence for defective T<>T replication in XP-V (45). This finding was made possible by making an essential improvement to the design of the T<>T-containing template that was used in our original T<>T replication assay. Highly selective detection of full-size, T<>T-containing daughter molecules was accomplished by the insertion of a latent restriction site (*Mfe*I) in the undamaged template strand opposite T<>T (Fig. 3).

1.3. Procedure

1.3.1. Preparation of T<>T-Containing, SV40 Origin-Containing Template

We prepared double-stranded template by the enzymatic elongation of a site-specific T<>T-containing oligonucleotide primer (28–29) annealed to circular, single-stranded DNA, with ligation, *in situ*. We scaled up the basic synthesis conditions as described in the site-directed mutagenesis protocol developed by Kunkel et al. (48), but extended the procedure to include methylation of GATC sites with Dam methylase. We also developed a carefully designed purification procedure that routinely yields tens of micrograms of pure, double-stranded template.

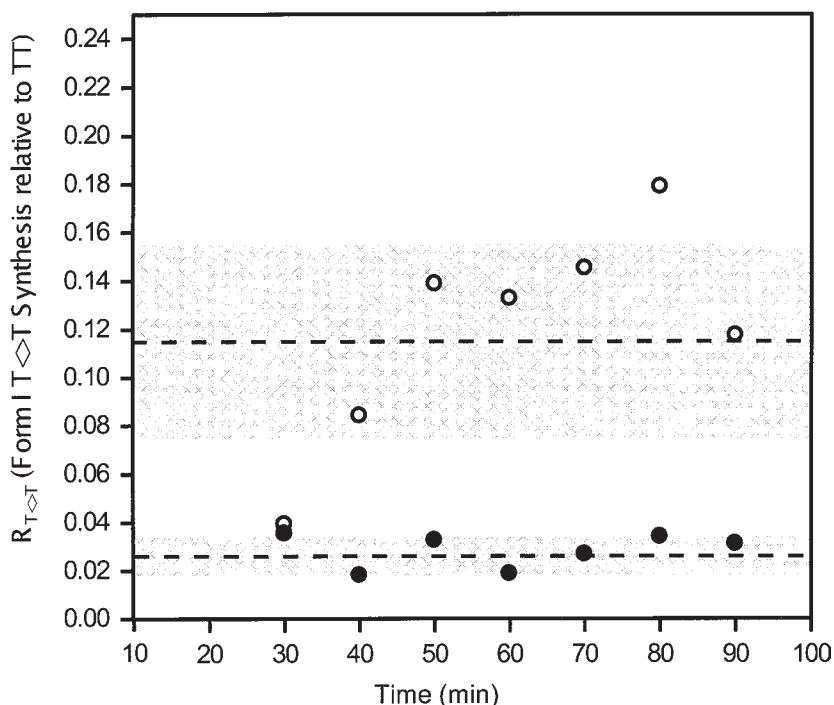


Fig. 2. Defective bypass replication of a UV-induced lagging strand cyclobutane thymine dimer in XP-V cell-free extracts. Comparison of bypass synthesis using normal fibroblast (VA13) and XP-V fibroblast (CTAG) as a function of time. Semisynthetic plasmid DNA molecules containing a single T<=>T dimer in the lagging strand were incubated with VA13 or CTAG cell-free extracts for 60 min, and labeled replication products were treated with T4 UV endonuclease followed by agarose-gel electrophoresis. Agarose-gel analysis of replication of undamaged and T<=>T-containing templates in vitro with VA13 and CTAG fibroblast cell extracts was performed as described (41–45). Labeled form I DNA containing T<=>T is nicked by T4 UV endonuclease and migrates as form II. The level of replicated T<=>T template is expressed as the ratio of the amount of T<=>T-specific replication (form $I_{T4 \text{ sensitive}}$) to the amount of replication with the control (TT) template ($R_{T \leftrightarrow T} = \text{form } I_{T4 \text{ sensitive}} \div \text{form } I_{TT}$). Comparison of this ratio between the two cell extracts gives a measure of relative competence in T<=>T bypass replication (41–45). Average values and standard deviation are indicated on dotted lines and shaded areas, respectively. Open circles, VA13 (control); filled circles, CTAG (XP-V).

1.3.2. SV40 Origin-Initiated Replication In Vitro

We used the site-specific T<=>T-containing SV40 replication template (and undamaged, TT-containing control template prepared in parallel) in replica-

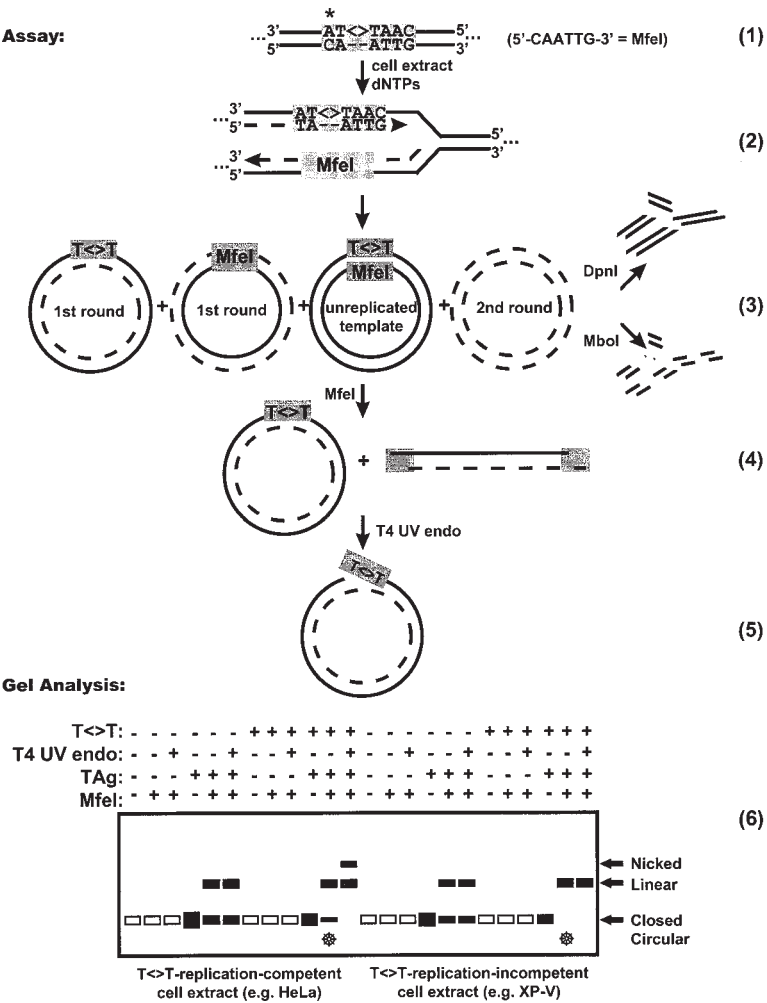


Fig. 3. SV40 origin-initiated replication in vitro. Digestion of the replication products by restriction enzymes reveals T◊T-containing form I DNA with high sensitivity.

tion assays that included an α -³²P-labeled nucleotide (dCTP), and commercially obtained TAg under conditions essentially as reported (13). Under these conditions, bidirectional, semiconservative synthesis yields labeled, first-round daughter molecules with hemimethylated GATC sites. These are distinguishable from parent (fully methylated), granddaughter (fully unmethylated), and subsequent-generation molecules (fully unmethylated) through the application of restriction enzymes specific for the methylation status of GATC sites. In

addition, by including a single mismatched basepair in the template, daughter and subsequent generation molecules derived from the undamaged template strand are selectively digested by *MfeI* restriction endonuclease (**Fig. 3**). In this way we can unambiguously detect daughter molecules derived from DNA synthesis on the T<>T-containing template strand.

1.3.3. Analysis of Replication Products

Labeled replication products are isolated after various incubation times and analyzed on ethidium-containing agarose gels or denaturing polyacrylamide gels following digestion with combinations of restriction enzymes and/or T<>T-specific nicking by T4 UV endonuclease V.

2. Materials

2.1. T<>T-Containing, SV40 Origin-Containing Template

2.1.1. 5'-Phosphorylated T<>T-Containing 20-Mer

1. T<>T-containing (or TT-containing control) 20-mer (*see Note 1*).
2. 100 mM ATP (Boehringer Mannheim, Indianapolis, IN).
3. T4 polynucleotide kinase (10 U/ μ L) and 10X kinase buffer (New England Biolabs, Beverly, MA).

2.1.2. Circular, Single-Stranded DNA with 20 Nucleotide Complementary Sequence and SV40 Origin Sequence

1. SV40 origin-containing phagemid with sequence corresponding to T<>T-containing primer (e.g., pKSoriMfeI(-) [**41**]). (*See Note 2*.)
2. LB medium with 100 μ g/mL tetracycline.
3. Bacterial culture plates: LB agar containing 100 μ g/mL ampicillin, 10 μ g/mL tetracycline.
4. M13K07 helper phage (Stratagene, La Jolla, CA; prepare stock as per supplier's protocol).
5. 2XYT medium containing 100 μ g/mL ampicillin, 70 μ g/mL kanamycin sulfate.
6. Molecular biology-grade phenol; phenol/chloroform/isoamyl alcohol (25:24:1); chloroform/isoamyl alcohol (24:1).
7. 3 M Sodium acetate, pH 5.2.
8. 95% Ethanol.
9. Electrophoresis-grade agarose.
10. Polyethylene glycol 8000 (Sigma, St. Louis, MO).
11. Sodium chloride.
12. 20 mg/mL proteinase K (Boehringer Mannheim).
13. Qiagen 2500, 500, and 100 columns.
14. Qiagen buffers: QBT, QC, and QF (Qiagen handbook).
15. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
16. Sephacryl 1000 SF resin (Pharmacia, Piscataway, NJ).

17. Ethidium bromide.
18. Dialysis tubing.
19. Spectrophotometer.

2.1.3. Second Strand Synthesis with Ligation

1. 10X Anneal buffer: 200 mM Tris-HCl, pH 7.4, 20 mM MgCl₂, 500 mM NaCl.
2. 50% Glycerol.
3. 10X Synthesis buffer: 5 mM dNTPs, 10 mM ATP, 100 mM Tris-HCl, pH 7.4, 50 mM MgCl₂, 20 mM dithiothreitol (DTT).
4. T4 DNA polymerase (Boehringer Mannheim).
5. T4 DNA ligase (5 U/μL; Boehringer Mannheim).

2.1.4. Methylation of GATC Sites and Final Purification

1. Dam methylase and 10X methylase buffer (NEB).
2. S-adenosylmethionine (NEB).
3. Restriction enzyme *DpnI* and 10X buffer #4 (NEB).
4. *Escherichia coli* exonuclease III (NEB).
5. Benzoylated naphthoylated DEAE (BND) cellulose (Sigma).
6. BND buffer: 20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 M NaCl.
7. T4 UV endonuclease V (gift from R. S. Lloyd, University of Texas).

2.2. SV40 Origin-Initiated Replication In Vitro

1. 10X SV40 reaction buffer: 300 mM HEPES, pH 7.8, 70 mM MgCl₂, 2 mM CTP, 2 mM GTP, 2 mM UTP, 40 mM ATP.
2. 20X dNTPs: 2 mM dATP, 0.2 mM dCTP, 2 mM dGTP, 2 mM TTP (UltraPure, Pharmacia).
3. 1 M Creatine phosphate (Boehringer Mannheim) in 20 mM HEPES, pH 7.8.
4. 2.5 mg/mL Creatine phosphokinase (Boehringer Mannheim) in 20 mM HEPES, pH 7.8.
5. 250 mM NaH₂PO₄ adjusted to pH 7.5 with NaOH.
6. α-³²P-dCTP (DuPont NEN Easytides; 6000 Ci/mmol).
7. SV40 large T-antigen (TAg) (Molecular Biology Resources, Milwaukee, WI).
8. Replication-competent cell extract (e.g., HeLa) (*see Note 3*).
9. 10% Sodium dodecyl sulfate (SDS).
10. ³H-labeled carrier DNA.
11. Sepharose 6B (Pharmacia).
12. Polyethyleneimine (PEI) cellulose thin-layer chromatography (TLC) plates (Machery-Nagel, Germany).
13. 0.5 M Ammonium bicarbonate.
14. Restriction enzymes *DpnI*, *MboI*, and *MfeI* (NEB).
15. Gel-fixing solution: 10% acetic acid, 10% methanol.
16. X-ray film and development facility or phosphorimager (e.g., Molecular Dynamics Model 400 PhosphorImager).

3. Methods

3.1. Preparation of T<>T-Containing, SV40 Origin-Containing Template

3.1.1. 5'-Phosphorylation of the T<>T-Containing 20-Mer

1. Combine 1000 pmol of T<>T-containing (or TT-containing control) oligonucleotide, 100 nmol of ATP, 10 μ L of the manufacturer-supplied 10X kinase buffer, deionized H₂O to 90 μ L, and 10 μ L (100 U) of T4 polynucleotide kinase.
2. Incubate at 37°C for 60 min.
3. Inactivate the enzyme by heating at 65°C for 20 min.

3.1.2. Preparation of Circular, Single-Stranded DNA with 20 Nucleotide Complementary Sequence and SV40 Origin Sequence

Prepare single-stranded DNA by superinfection of *E. coli* XL1-Blue carrying the plasmid (e.g., pKSoriN[+/-] or pKSoriMfe[-]) with helper phage M13K07. Single-stranded DNA of the highest purity is essential in obtaining a good yield of semisynthetic double-stranded DNA in subsequent steps. We have obtained consistently good yields and purity by strict adherence to the following protocol:

1. Inoculate several (to ensure that at least one dense culture is obtained the next day) 10-mL overnight cultures (LB + 100 μ g/mL ampicillin, 10 μ g/mL tetracycline) with colonies from a fresh plating (LB/agar + 100 μ g/mL ampicillin, 10 μ g/mL tetracycline) of plasmid-transformed bacteria.
2. Use 4 mL of a dense overnight culture to inoculate 200 mL of LB/ampicillin/tetracycline, and shake for 0.5–2 h at 37°C to a density of 0.1 OD₆₀₀ ($\sim 5 \times 10^5$ cells/mL).
3. Superinfect with helper phage M13K07 at a multiplicity of infection (MOI) of 5.
4. After 1 h of incubation with shaking at 37°C, dilute the superinfected culture into prewarmed 2XYT medium containing 100 μ g/mL ampicillin and 70 μ g/mL kanamycin sulfate (25 mL into each of 6 2-L flasks containing 500 mL of medium to achieve good aeration).
5. Shake vigorously (250 rpm shaker speed) at 37°C for 12–18 h (max.).
6. Pellet the bacteria by centrifuging twice at 5000g for 15 min at 4°C.
7. Prepare a small sample (300 μ L) of DNA for analysis by extracting the supernatant with phenol (300 μ L), then with phenol/chloroform (300 μ L), and finally with chloroform (300 μ L); add 30 μ L of 3 M NaOAc, pH 5.2, 750 μ L of 95% ethanol, and centrifuge the DNA in a microcentrifuge (12,000g).
8. Run the sample on an agarose gel containing ethidium bromide together with some standard samples to estimate the yield of single-stranded DNA (3 L of culture should produce 2–5 mg).
9. Precipitate the phage particles by adding 20 g of polyethylene glycol 8000 and 15 g of NaCl for each 500 mL of supernatant, stirring at room temperature for an additional 30 min after complete dissolution of the PEG and NaCl.
10. Pellet the phage at 10,000g for 20 min at 4°C.

11. Decant the supernatant, and drain the pellets thoroughly, but not to dryness, carefully wiping any liquid adhering to the mouth of the inverted centrifuge bottles.
12. Resuspend the pellets in 2×5 mL of 10 mM Tris-HCl (pH 8.0) for each 500 mL of supernatant, being careful to collect all of the pellet, since it will be smeared out over a large area on the wall of the centrifuge bottle.
13. Add 1 mL of proteinase K (20 mg/mL) to the combined resuspended pellets (>60 mL). Incubate at 50°C for 3–4 h.
14. Prepare a Qiagen 2500 column(s) by equilibration with buffer QBT, and apply the digestion mixture, diluted 1:1 with QBT.
15. Wash and elute the column with buffers QC and QF, and precipitate the DNA as directed in the Qiagen handbook.
16. Resuspend the DNA in 2–5 mL of TE buffer as needed.
17. Separate the single-stranded phagemid DNA from contaminating helper phage DNA (and possibly a small amount of double-stranded plasmid and chromosomal DNA from cell breakage): apply the DNA sample to a 2.6×70 cm column of Sephacryl S1000 SF, equilibrated in TE, pH 8.0, containing 1 M NaCl, and elute the column, collecting 5-mL fractions (a 3.2-kb phagemid elutes after ca. 275–300 mL).
18. Determine the absorbance at 260 nm for each fraction, and analyze the DNA-containing fractions on an agarose gel containing ethidium bromide.
19. Combine fractions containing pure single-stranded phagemid DNA, and dialyze against TE (pH 8.0).
20. Determine the concentration of single-stranded DNA from the A_{260} (1 absorbance unit ≈ 38 μ g of single-stranded DNA).

3.1.3. Second-Strand Synthesis with Ligation

First, determine the optimal primer: template ratio, and template concentration by performing analytical-scale syntheses.

1. Anneal the phosphorylated primer to the template: combine 5 μ L of 10X anneal buffer, 0.5 pmol (~ 0.5 μ g of a 3-kb phagemid) of single-stranded template, 0, 1, 2, 4, 8, and 12 pmol of phosphorylated T<>T-containing or undamaged control 20-mer, and deionized H₂O to 50 μ L.
2. Place the tubes in a heating block at 70°C for 5 min, followed by cooling on the bench to <37°C.
3. Spin down the condensation in a microcentrifuge, and chill on ice.
4. Add, in order, 1 μ L of 50% glycerol, 5 μ L of 10X synthesis buffer, 5 μ L of T4 DNA polymerase, and 4 μ L of T4 DNA ligase; mix well. Incubate on ice for 5 min, at room temperature for 5 min, and at 37°C for 90 min.
5. Inactivate the enzymes at 65°C for 20 min.
6. Analyze ~ 100 ng samples on an ethidium bromide-containing agarose gel using double-stranded plasmid and single-stranded template as size markers.

Conversion to form I should be highly efficient under optimal conditions ($\sim 80\%$ by visual inspection). Choose the optimal primer:template ratio, and

perform additional analytical-scale syntheses, varying the single-stranded template concentration, e.g., 0.5, 0.75, 1.0, 1.5, 2.0 pmol to finalize the optimization before continuing on to preparative scale syntheses. Increase to preparative scale. We use 10 times the analytical scale in each of 10 1.5-mL microcentrifuge tubes. In our hands, this yields 25–50 µg of very pure final product. This is an “all-or-nothing” commitment of large quantities of expensive commercial enzymes. Be careful! Analyze a sample from each tube on an agarose gel containing ethidium bromide before proceeding. Combine the synthesis mixtures, dilute 1:1 with QBT, and apply to an appropriate size Qiagen column, pre-equilibrated with QBT. Wash, elute, and precipitate the DNA as described in **step 15, Subheading 3.1.2.**

3.1.4. Methylation of GATC Sites and Final Purification

Double-stranded, semisynthetic plasmid DNA is hemimethylated at the *E. coli* Dam modification site (N^5 of adenine of GATC). Methylation is accomplished by direct application of commercially available Dam methylase together with *S*-adenosylmethionine. After methylation, the purification procedure consists of an enzymatic step, followed by the selective adsorption of molecules containing single-stranded gaps (produced by the enzymatic treatments) to benzoylated-naphthoylated DEAE cellulose, which allows the isolation of highly purified, circular double-stranded DNA. Finally, the presence of T<>T is confirmed by complete conversion of an aliquot of the purified product to form II (nicked) by treatment with T4 UV endonuclease.

1. Resuspend the pellet in Dam methylase buffer to a final concentration of ~126 µg/mL.
2. Add Dam methylase to a final concentration of 100 U/mL and *S*-adenosylmethionine (must be fresh) to a final concentration of 100 µg/mL. Incubate for 2 h at 37°C.
3. Confirm methylation by digestion with *DpnI*, being certain to supplement the reaction buffer (NEB #4) with 200 mM NaCl.
4. Repeat the methylation step if necessary (often two methylation treatments are needed).
5. Inactivate the enzyme at 65°C for 20 min. Remove an aliquot for gel analysis (**step 7**).
6. Add 10 U of *E. coli* exonuclease III/µg of DNA. Incubate for 1 h at 37°C. (*See Note 4.*)
7. Check the digestion on an agarose gel containing ethidium bromide using the aliquot reserved from **step 5** as a control; two bands (double-stranded circular and single-stranded circular), and possibly a faint smear, should be visible.
8. Isolate the DNA using a Qiagen column as described in **steps 14–16, Subheading 3.1.2.**
9. After resuspending the DNA pellet in TE (pH 8.0), adjust the buffer to 20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, and 1 M NaCl (i.e., the same as BND buffer),

with a final volume corresponding to 200 μL for every 100 μg of initial single-stranded template.

10. Pack a column of benzoylated naphthoylated DEAE cellulose, equilibrate in BND buffer (400 μg for every 100 μg of initial single-stranded template), and wash the column until the wash buffer is free of UV absorbance, monitoring the spectrum between 200 and 400 nm.
11. Apply the DNA to the column, collect 0.5-mL fractions, and monitor the absorption spectrum (200–400 nm) for DNA.
12. DNA containing single-stranded regions binds tightly to the column, whereas double-stranded DNA elutes. Combine fractions containing significant absorbance, and calculate the yield from the absorbance of the combined fractions at 260 nm.
13. Combine 25–100 ng of T<>T-containing DNA in T4 UV endonuclease buffer, and add 1 ng of T4 UV endonuclease V. Incubate at 37°C for 30 min.
14. Analyze on an agarose gel containing ethidium bromide. We observe “100%” conversion of closed-circular T<>T-containing DNA to nicked form. We also observe that the undigested plasmid is “nick-free.”

3.2. SV40 Origin-Initiated Replication In Vitro

3.2.1. Assembly of Reaction Components

Depending on the analytical method after replication, and the efficiency of the cell extract, we have performed replication reactions on scales from 12.5 to 150 μL .

1. For a 12.5- μL reaction, combine:
 - 1.25 μL 10X SV40 reaction buffer.
 - 0.625 μL 20X dNTPs.
 - 0.5 μL 1 M creatine phosphate.
 - 0.5 μL 2.5 mg/mL creatine phosphokinase.
 - 0.75 μL 250 mM NaH_2PO_4 (pH 7.5).
 - 0.375 μL α - ^{32}P -dCTP.
 - 0.5 μg SV40 TAg.
 - 1.5 ng plasmid DNA.
 - 62.5 μg cell extract.
 - Deionized H_2O to 12.5 μL .
2. Incubate at 37°C for the desired length of time. When performing a time-course, increase the scale of the reactions, and remove aliquots at different times for analysis.
3. To stop the reaction, add 1 μL of 10% SDS, 5 μL of ^3H -labeled carrier DNA (*see Note 5*), and 0.2 μL of 10 mg/mL proteinase K. Incubate at 50°C for 30 min.

3.2.2. Purification of DNA from the Reaction Mixture

1. Apply the proteinase K-treated reaction mixture to a 1-mL column of Sepharose 6B equilibrated in a buffer suitable for the subsequent enzymatic analysis step (e.g., for *MfeI* analysis, we use NEB buffer #4).

2. Wash the column with 300 μL of the appropriate buffer in aliquots of 2×25 , 1×100 , and 1×150 μL .
3. Collect the 150- μL fraction; this should contain $\sim 90\%$ of the incorporated label.
4. Confirm the removal of unincorporated label by PEI cellulose TLC using 0.5 *M* ammonium bicarbonate as elution buffer; labeled DNA remains at the origin, whereas labeled mononucleotides migrate with $R_f \sim 0.5$. If desired, count an aliquot for quantitation of absolute nucleotide incorporation using the ^3H counts for normalization of the ^{32}P .

3.2.3. Analysis of Form I Replication Products Using the Latent *MfeI* Restriction Site

1. Aliquot 20 μL of column-purified replicated DNA into each of two 1.5-mL microcentrifuge tubes. Label one tube “+*MfeI*” and the other “–*MfeI*.”
2. To the “+” tube, add 0.5 U of *MboI* and 2 U of *MfeI*.
3. To the “–” tube, add 0.5 U of *MboI*.
4. Incubate at 37°C for 60 min.
5. Inactivate the enzymes at 65°C for 20 min.
6. Add 1 μL of NEB buffer #4 and 3 μL of 2 *M* NaCl to each tube.
7. Add 0.75 U of *DpnI* to each tube.
8. Incubate at 37°C for 60 min.
9. Inactivate the enzymes at 65°C for 20 min.
10. Add gel-loading buffer to each sample.
11. Count 5 μL in a scintillation counter using the ^3H channel.
12. Electrophorese on a 0.8% agarose gel containing ethidium bromide until the bromophenol blue marker reaches the end of the (20-cm) gel.
13. Fix the gel in 10% acetic acid, 10% methanol solution, shaking gently for 30 min.
14. Wash the fixed gel in H_2O for 15 min.
15. Dry the fixed gel, and expose to film or a phosphorimager plate overnight.

3.3. Quantitation and Calculation

The improved design of the *MfeI*-based replication assay greatly enhances the signal-to-noise ratio by reducing the number of background subtractions involved in calculating the amount of fully replicated T<>T-containing template. Highly selective detection of full-size, T<>T containing daughter molecules is achieved by the insertion of a latent restriction site (*MfeI*) in the undamaged template strand, opposite T<>T. Digestion with *MfeI* linearizes daughter molecules synthesized from the undamaged parent strand. This leaves as the only label-containing closed-circular (form I) DNA, daughter molecules synthesized from the T<>T-containing parent strand. This is then confirmed by digestion with T4 UV endonuclease, which specifically breaks DNA strands at pyrimidine dimers. Additionally, the presence or absence of bypass can be determined on visual inspection of autoradiographs. The presence of a single band in the *MfeI*-treated lane immediately indicates bypass.

The absence of this band unequivocally reveals the inability of a cell extract to perform bypass replication of T<>T, as demonstrated in the case of XP-V. In fact, this assay is particularly suited to the detection of in vitro complementation through the addition of column fractions prepared from HeLa cell extracts to XP-V extract.

Dried gels are exposed to phosphorimaging plates, which are then scanned as 16-bit images using a Molecular Dynamics PhosphorImager with Image Quant software. Relative ratios of form I and form II DNA are measured in the presence and absence of T4 UV endonuclease to determine the fraction of product form I molecules containing T<>T. In the case of the latent *MfeI*-containing template, form I intensity (*I*) is measured before and after digestion with *MfeI*, with T4 UV endonuclease merely providing confirmation of the identity of the form I DNA after *MfeI* digestion. Background owing to nonreplicative incorporation of label, determined from reactions without TAg, is then subtracted. Comparisons between control (undamaged), leading strand T<>T, and lagging strand T<>T refer to relative rates of accumulation of the specified product DNA molecules. Relative rates are calculated from slopes of plots of the intensities of gel bands in the linear region of time-course experiments as determined by linear least-squares regression analysis. These rates are based on pixel values and apply to relative comparisons within each experiment.

3.3.1. Efficiency of Replicative Bypass

The conversion of form I molecules to form II by T4 UV endonuclease is used to calculate the amount of replication of templates containing T<>T:

$$I_{T<>T} = 2 \times I_{T4\text{-sensitive}} \quad (1)$$

where $I_{T<>T}$ = fully replicated T<>T-derived form I product and $I_{T4\text{-sensitive}}$ = form I product nicked by T4 UV endonuclease or, when *MfeI* is used to determine the level of replicated T<>T template, $I_{T<>T} = 2 \times (I_{\text{total}} - I_{MfeI})$, where I_{total} = total form I product and I_{MfeI} = form I product remaining after *MfeI* digestion.

The replicated T<>T-containing template equals two times the T4 UV endonuclease-sensitive form I or two times the *MfeI*-sensitive form I, because only one of the two daughter molecules produced from a single parent molecule contains T<>T. The slope of a plot of $I_{T<>T}$ as a function of time gives the rate of replication of a T<>T-containing template.

The efficiency of T<>T replication is then expressed as the ratio of $I_{T<>T}$ bands to the total form I DNA synthesized as follows:

$$B = I_{T<>T} / I_{\text{total}} \quad (2)$$

where B = fraction of form I with a bypassed T<>T dimer.

3.3.2. Efficiencies of Fork Uncoupling and Gap Formation

3.3.2.1. LEADING STRAND T<>T

The amount of uncoupling (synthesis templated by the undamaged strand in the absence of completion of synthesis of the T<>T containing strand) is derived from the form I product DNA free of T<>T:

$$I_U = I_{\text{total}} - I_{T<>T} \quad (3)$$

where I_U = form I DNA from uncoupled synthesis of the undamaged strand. This quantity is the undamaged form I DNA in excess of that accounted for by the complete replication of T<>T-containing parent molecules. The efficiency of fork-uncoupling from replication is then expressed as the following ratio:

$$U = (I_{\text{total}} - I_{T<>T})/I_{\text{total}} \quad (4)$$

or

$$U = 1 - (I_{T<>T}/I_{\text{total}}) \quad (5)$$

where U = relative amount of form I DNA from uncoupled synthesis of the undamaged strand.

The slope of a plot of U as a function of time gives the rate of accumulation of uncoupled replication product.

3.3.2.2. LAGGING STRAND T<>T

When applied to synthesis using the lagging T<>T template, Eqs. 3, 4, and 5 indirectly yield the rate and efficiency of formation of gapped molecules as follows:

$$I_G = I_{\text{total}} - I_{T<>T} \quad (6)$$

and

$$G = 1 - (I_{T<>T}/I_{\text{total}}) \quad (7)$$

where I_G = form I DNA from gapped synthesis of the undamaged strand and G = relative amount of form II from gapped molecule synthesis of the damaged strand.

3.3.3. Efficiency of Replicative Inhibition

The relative level of blockage owing to the T<>T dimer on leading or lagging strands can be derived from the difference between the total amount of form I synthesized and the amounts of form I derived from T<>T bypass and fork uncoupling or gap formation, respectively, using the following equations:

1. For leading strand T<>T:

$$\% \text{ Inhibition} = 100 - \%(\text{bypass} + \text{uncoupling}) \quad (8)$$

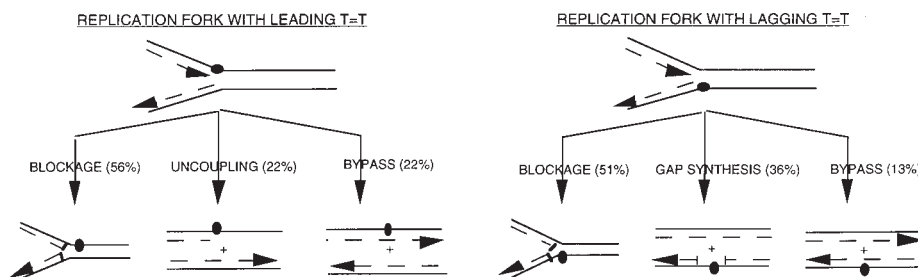


Fig. 4. Differential replication of a *cis-syn* cyclobutane thymine dimer, $T \diamond T$, in leading and lagging strands of a SV40-based replicon incubated in replication-competent HeLa cell-free extracts. The probability of occurrence of each event is indicated as a percentage. Some forks are able to bypass the leading or lagging $T \diamond T$ completely with a slightly higher efficiency along the leading strand, i.e., 22 vs 13%. In addition, strong blocks to fork progression are induced by both the leading or lagging $T \diamond T$, but unusual replication intermediates, such as uncoupled forks with the leading $T \diamond T$ and formation of single-strand gaps with the lagging $T \diamond T$, are also detected. See ref. 41 for experimental details.

2. For lagging strand $T \diamond T$:

$$\% \text{ Inhibition} = 100 - \%(\text{bypass} + \text{gap formation}) \quad (9)$$

3.4. Conclusions and Future Prospects

Recent biochemical studies with human cell-free extracts and circular viral minireplicons have started to dissect the molecular structure of replication forks encountering a DNA lesion on the leading or lagging strands (Fig. 4). DNA damage located on the leading strand blocks the synthesis of both strands; although reinitiation does not occur downstream from the damaged site on either strand, completion of undamaged lagging strand indicates the possibility of uncoupling of strand synthesis at the replication fork. DNA damage on the lagging strand interrupts completion of the Okazaki fragment spanning the damaged site; however, synthesis of additional Okazaki fragments downstream from the lesion occurs, allowing fork progression behind a gapped duplex. Figure 4 illustrates the respective efficiencies of these alternative steps as measured with a unique *cis-syn* cyclobutane thymine dimer, i.e., $T \diamond T$ (41).

Genetic analysis of mutants isolated from eukaryotic model systems, such as yeast and fruitfly, indicates the coexistence of error-prone and error-free pathways for the replication of DNA damage (1). In vitro replication studies with the human cell-free system demonstrate the occurrence of error-prone

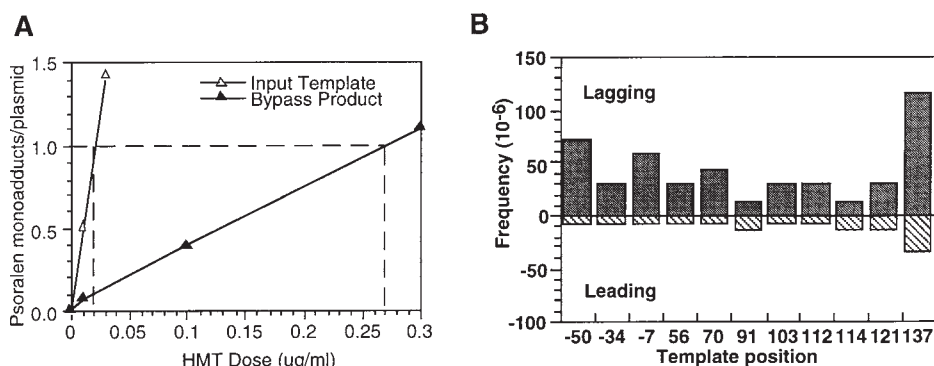


Fig. 5. (A) Efficacy of bypass replication of 4'-hydroxymethyl-4,5',8-trimethyl psoralen (HMT) MA on SV40-based plasmid incubated in replication-competent HeLa cell-free extracts. Average number of psoralen MA calculated by Poisson analysis in unreplicated (open triangle) and replicated (filled triangle) plasmid DNA. (Adapted with permission from (49)). (B) Strand-specificity of psoralen-photo-binding-induced minus-one frameshifts at template T residues by position of an SV40-based template incubated in replication-competent HeLa cell-free extracts. Position +1 is the first transcribed base of the lacZalpha gene. Adapted with permission from (49).

translesion replication in mammalian cells along both the leading and lagging strands. For example, we observed that approximately one out of nine monoadducts (MA) induced by psoralen and UV-A light was bypassed by the cell-free human extracts made from HeLa cells (Fig. 5A). Such a bypass replication was accompanied by an increase in targeted mutations, i.e., substitutions and deletions occurring in runs of thymines, owing to error-prone translesion synthesis opposite MAs. Interestingly, a strand bias in single A-T deletions was observed in favor of the lagging strand, indicating a potential asymmetry of the error-prone bypass replication machinery operating on the leading and lagging strands (Fig. 5B). Such a mutagenic pathway may involve a known DNA polymerase, such as DNA polymerase δ , or a new and specific DNA polymerase(s).

On the other hand, the high persistence of DNA damage in mammalian cells also implies the existence of an error-free pathway(s) for efficient replication of DNA damage. One of us has previously proposed that two distinct repair pathways may operate in mammalian cells, one for each strand of DNA (1). With lesions on the lagging strand, gaps smaller than 200 bases resulting from blocked Okazaki fragments would be filled by general recombination using the homologous newly-replicated duplex in a process analogous to bacterial postreplication repair. With lesions on the leading strand, the stalled DNA poly-

merase would bypass the blocking lesion by switching from the damaged template to the newly replicated complementary lagging strand to copy the (last) Okazaki fragment offshoots.

The identification of an excision repair-proficient human syndrome, such as XP-V, with a specific defect in bypass DNA replication (45) is an encouraging step toward the goal of elucidating the pathways and molecular steps of such a poorly understood process in mammalian cells. Since the bypass deficiency of a leading strand T<>T in several XP-V extracts is quite severe, i.e., more than a 10-fold reduction in bypass efficiency relative to control extracts, such an experimental system appears suitable to develop a strategy for the in vitro complementation of XP-V with fractionated bypass-proficient cell-free extracts and isolate the functional XPV factor. The function of the XPV protein may be rather specific to the DNA replication machinery, e.g., a cofactor of a DNA polymerase, such PCNA or RF-C. As discussed elsewhere (1), such an in vitro deficiency in translesion replication of XP-V will have to be reconciled with the in vivo evidence that XP-V is defective in an error-free pathway of replication of DNA damage (50). Therefore, the XPV mutation may decrease the efficiency of translesion replication, i.e., probability of replication past a lesion, but increase its mutagenicity, i.e., probability of misincorporation per bypassed lesion. Alternatively, both error-prone and error-free pathways of replication of DNA lesions are operative in the SV40-based human cell-free extracts, and XPV carries a defect in the latter, but not the former.

4. Notes

1. It is beyond the scope of this chapter to describe in detail the preparation of DNA oligomers carrying a single, site-specific T<>T. Briefly, a T<>T phosphoramidite is prepared essentially as published (47,48). Then, the oligomer is synthesized by standard, machine-automated methodology by including the T<>T amidite in an extra reservoir on the machine, and including an appropriate coupling step in the desired sequence. With the HPLC-purified oligonucleotide in hand, phosphorylation is a straightforward application of the manufacturer-recommended protocol included with the T4 polynucleotide kinase.
2. To construct this phagemid, we used the *Hind*III (5171)-*Pvu*II (270) fragment of SV40, which was blunt-end-ligated to a *Bam*HI linker (5'-pCCGGATCCGG), and cut with *Bam*HI to yield a *Hind*III-*Bam*HI, SV40 origin fragment. This was cloned into the unique *Bam*HI and *Hind*III sites of pBluescript II KS (+) and pBluescript II KS (-) to yield the SV40 origin-containing phagemid, pKSori(+/-) (41). Insertion of the 20 nt sequence (GCTCGAGCTCAATTAGTCAG) corresponding to the T<>T-containing 20-mer was obtained by site-directed mutagenesis according to the method of Kunkel et al. (41). By using 60 nt primers annealed to uracil-containing single-stranded pKSori(+/-), the resulting 20 nucleotides bulge produced a 20 nt insertion (pKSoriN[+/-]) after transfection into an

- ung+* *E. coli* strain (41). Finally, through another round of site-directed mutagenesis, we made a single nucleotide change to generate pKSoriMfe(-) carrying a (latent) *MfeI* cognate sequence at the T<>T site (45) (Fig. 3).
3. HeLa cell cytoplasmic extract is prepared from suspension cultures according to Stillman and Gluzman (13). Harvest 1 L of cells ($\sim 5\text{--}6 \times 10^6$ cells/mL) by low-speed centrifugation and wash in phosphate-buffered saline. Wash the cells in 10 mL of ice-cold hypotonic buffer (20 mM HEPES-KOH, pH 7.5, 5 mM KCl, 1.5 mM MgCl_2 , and 0.1 mM DTT), and then resuspend in 5 mL of hypotonic buffer and incubate on ice for 10 min. Disrupt the cells with 20 strokes of a Dounce homogenizer (B pestle), and incubate on ice for 30 min. Centrifuge the suspension at 16,000g for 10 min. Divide the supernatant into aliquots, and store at -70°C .
 4. *E. coli* exonuclease III catalyzes the removal of nucleotides from 3'-OH termini. It is specific for duplex DNA with blunt or recessed 3' ends, but also acts at nicks to produce gaps.
 5. The ^3H -labeled carrier DNA serves as a marker to monitor relative DNA recovery among the various samples.

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Detection of Chromatin-Bound PCNA in Cultured Cells Following Exposure to DNA-Damaging Agents

Masahiko Miura and Takehito Sasaki

1. Introduction

It has been a decade since proliferating cell nuclear antigen (PCNA) was found to be essential for DNA replication as an auxiliary protein of DNA polymerase δ (*1,2*; reviewed in *3,4*). There is compelling evidence that a homotrimer of PCNA forms a toroidal clamp around duplex DNA (*5–7*), an association catalyzed by replication factor C (RF-C) in an ATP-dependent manner (*8*). The PCNA clamp binds to polymerase δ and stimulates its processivity by sliding along the DNA template. PCNA similarly enhances the activity of another DNA polymerase, ϵ , under certain conditions in vitro (*9*). PCNA is now also known to be an indispensable factor in nucleotide excision repair (NER) (*10*) and in an alternative pathway of base excision repair (*11,12*). Thus, detection of chromatin-associated PCNA in cells exposed to DNA-damaging agents is indicative of DNA repair activity, provided the cells are quiescent or at least in G1 or G2 phase. One may, however, encounter a problem in detecting such a PCNA clamp, because chromatin-unbound PCNA is also present within nuclei.

This chapter describes two different immunostaining methods to detect chromatin-bound PCNA in UV-irradiated quiescent cells. One method uses an anti-PCNA monoclonal antibody (MAb), PC10 (*ref. 13*), which is commercially available from several sources. The other method employs a unique anti-PCNA autoantibody, AK, derived from an autoimmune disease patient (*14*). The former requires a detergent extraction step to remove chromatin-unbound PCNA prior to fixation (*Fig. 1*), but the latter does not (*15*). UV irradiation induces chromatin-bound PCNA associated with the NER process in cells and can be visualized with these methods.

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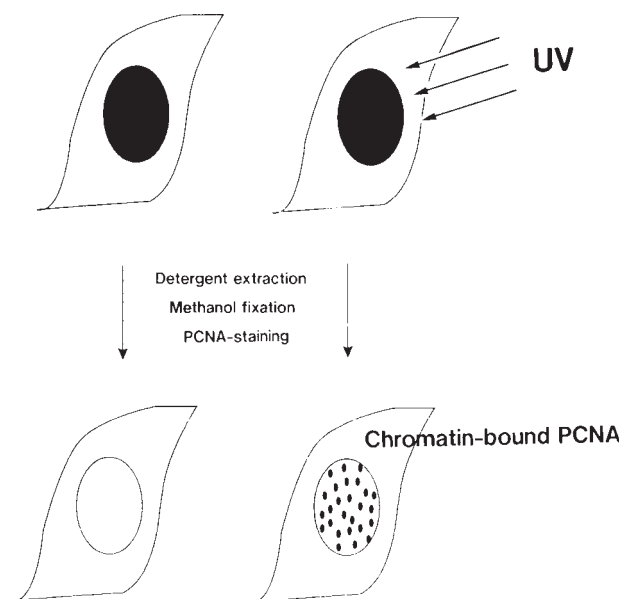


Fig. 1. Schematic diagram of the protocol for PCNA staining using MAb PC10.

2. Materials

2.1. Cell Culture

1. Lab-Tek chamber slides (eight-well) (Nunc, Naperville, IL).
2. Primary human fibroblasts.
3. Growth medium (e.g., Eagle's modified minimum essential medium) supplemented with 10% fetal bovine serum (FBS).

2.2. UV Irradiation

1. Germicidal lamp (e.g., Toshiba, Tokyo, Japan).
2. UV dosimeter.

2.3. Immunofluorescence Staining

1. Dulbecco phosphate-buffered saline (DPBS) without calcium or magnesium: 8.0 g NaCl, 0.2 g KCl, 1.44 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.2 g KH_2PO_4 in 1 L of distilled water, pH 7.4.
2. Millonig phosphate buffer (MPB): 8.5 g NaCl, 0.43 g NaOH, 2.12 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ in 1 L of distilled water, pH 7.4.
3. 0.5% Triton X-100 in DPBS supplemented with 1% bovine serum albumin (BSA).
4. 0.3% Triton X-100 in MPB.
5. 100% Methanol.
6. Anti-PCNA MAb PC10 (e.g., Calbiochem, Cambridge, MA) or anti-PCNA auto-

Table 1
Comparison of the PCNA-Staining Characteristics of α -PCNA Antibodies PC10 and AK Following Various Fixation Methods

Fixation method	Antibodies	
	PC10	AK
4% Formaldehyde alone	U + B ^a	U + B
Detergent extraction + 4% formaldehyde	B ^b	B
Methanol alone	U + B	B ^c
Detergent extraction + methanol	B ^c	B
Acetone alone	U + B	B

^aU + B indicates that both chromatin-unbound and bound PCNA can be visualized.
^bB indicates that only chromatin-bound PCNA can be visualized.
^cThese methods are described in the text.

antibody AK (**14**) obtained from an autoimmune patient (not commercially available) (*see* **Note 1**).

7. Fluorescein isothiocyanate- (FITC-) conjugated antimouse IgG (for PC10) or FITC-conjugated antihuman IgG antibody (for AK).
8. 95% Glycerin in distilled water.
9. Cover slips.
10. Immunofluorescence microscope.

3. Methods

Chromatin-unbound and bound PCNA can be visualized as shown in **Table 1**, depending on anti-PCNA antibodies, fixation methods, detergent extraction, and their combinations. Representative methods shown by bold letters in Table 1 will be described to detect only the chromatin-bound PCNA following UV-irradiation.

3.1. Cell Culture

Plate 10⁴ cells (in 500 μ L of growth medium) in four wells of an eight-well Lab-Tek chamber slide (*see* **Note 2**) and make them quiescent by incubating them in the same medium for 7–8 d (*see* **Note 3**).

3.2. Measurement of UV Dose

1. Measure the UV dose under a germicidal lamp using a UV dosimeter (*see* **Note 4**).
2. Calculate the exposure time to obtain the required dose.

Caution: UV radiation is very dangerous, especially to eyes.

3.3. Immunofluorescence Staining of PCNA Using PC10

1. Wash the cells twice with 300 μ L of DPBS, and aspirate the overlaying buffer (*see* **Note 5**).

2. Irradiate the cells with UV light (10–40 J/m²) (*see Note 6*).
3. Gently add 300 μ L of fresh growth medium to each well, and incubate the cells at 37°C for 30 min to allow DNA repair (*see Note 7*).
4. Wash the cells twice with DPBS, and very gently add DPBS containing 0.5% Triton X-100 and 1% BSA. Incubate at 4°C for 10 min (*see Note 8*).
5. Very carefully remove the plastic separators and gum linings on a paper towel (*see Note 9*).
6. Gently immerse the slides in DPBS for 10 s.
7. Drain off the DPBS, and immerse the slides in methanol at –20°C for 5 min.
8. Rinse in three changes of MPB, and drain it off. Do not allow the cells to dry completely.
9. Cover the four wells with 500 μ L of PC10 (diluted 1:50 in MPB containing 0.3% Triton X-100), and incubate at room temperature for 30 min. (*See Notes 10 and 11.*)
10. Rinse in three changes of MPB.
11. Cover the four wells with 500 μ L of FITC-conjugated antimouse IgG (diluted 1:50–500 in MPB containing 0.3 % Triton X-100), and incubate at room temperature for 30 min. (*See Note 12.*) Keep the slides in the dark.
12. Rinse in three changes of MPB, and drain it off. Allow the slides to dry briefly.
13. Cover the four wells with 50 μ L of 95% glycerin.
14. Mount the cover slips, and remove bubbles and excess glycerin.
15. Observe the cells with a fluorescence microscope under blue-light excitation (405, 435, and ~490 nm, B-excitation by Olympus). (*See Notes 13 and 14.*)

3.4. Immunofluorescence Staining of PCNA Using AK Serum

1. Follow **steps 1–3 of Subheading 3.3.**
2. Very carefully remove the plastic separators and gum linings on a paper towel (*see Note 9*).
3. Drain off the growth medium.
4. Immerse the slides in methanol at –20°C for 5 min.
5. Rinse in three changes of MPB, and drain it off. Do not allow the cells to dry completely.
6. Cover the 4 wells with 500 μ L of AK serum (diluted 1:500 in PBS containing 0.3% Triton X-100) and incubate at room temperature for 30 min. (*See Note 15.*)
7. Rinse in three changes of MPB, and drain it off.
8. Cover the four wells with 500 μ L of antihuman IgG (diluted 1:50–500 in 0.3 % Triton X-100 solution), and incubate at room temperature for 30 min. Keep the slides in the dark.
9. Follow **steps 12–15 of Subheading 3.3.** (*see Note 16*).

4. Notes

1. AK, but not most, anti-PCNA antibodies recognize conformation-dependent epitopes of PCNA and are able to neutralize PCNA-dependent activities of DNA polymerase δ completely in vitro (*16*). Some other autoantibodies against PCNA may be used instead of AK (*17*).
2. We usually use only four wells of the eight-well slides to allow easy handling.

3. The percentage of S-phase cells should be as low as possible in this experiment, because cells undergoing DNA replication also stain positive for PCNA. Primary diploid fibroblasts easily become quiescent in plateau phase even in 10% FBS growth medium. After 7–8 d, cultures usually show <1% of S-phase cells.
4. The dose rate should be adjusted by changing the distance from the lamp so that irradiation finishes within 2 min. This ensures that the cells stay wet without the need for overlaying medium, which would block UV penetration.
5. Do not completely aspirate the DPBS. A small amount of DPBS should be left at the edges of the plastic separators to keep the cells wet.
6. 10 J/m² of UV-C usually gives appropriate staining intensity for most experiments.
7. Staining intensity reaches a peak about 30 min after irradiation, and then gradually decreases. It returns to almost background levels 24–48 h after irradiation.
8. Cells tend to be easily detached from the slide after this step. Careful attention should be paid to handling the slides thereafter.
9. Avoid aspirating the entire solution.
10. A blocking step is not usually required. Several different dilutions of antibody should be tried to obtain optimum staining conditions in your own experiment. Diluted antibody solutions can be stored in the dark at 4°C for up to 1 mo.
11. PC10 conjugated with FITC is now commercially available from Leinco Technologies, Inc. (Ballwin, MO). When this is used, **steps 11** and **12** can be omitted.
12. The diluted solution can be stored in the dark at 4°C for up to 1 mo.
13. Slides can be kept in the dark at 4°C before analysis for up to several hours.
14. Using this method, we have detected chromatin-bound PCNA in fibroblasts following exposure to 5–30 Gy of X-irradiation (**18**) and after 1 h of treatment with 100 μ M cisplatin (**19**), or 100 μ M mitomycin C (Miura et al., unpublished data).
15. Diluted solution can be stored in the dark at 4°C for at least up to 1 mo.
16. In general, this method shows a somewhat higher background, but strong staining intensity for positive nuclei can be obtained. A particular advantage of this method is that cell morphology can be well retained because the detergent extraction step is not required.

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Induction of p53 Protein as a Marker for Ionizing Radiation Exposure In Vivo

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1. Introduction

The use of ionizing radiation as a therapeutic agent has been recognized for almost a century, and continues to be widely used for the treatment and palliation of many human cancers. Ionizing radiation can also be mutagenic or lethal to individual cells, thus a critical balance must be achieved when using radiation as a form of anticancer treatment to ensure tumor cell death with minimal side effects to normal tissue and organ function. Morphological damage following nonlethal exposure to whole-body ionizing radiation is detectable only in a few select cell types. Histological studies on tissue derived from irradiated mammals reveal immediate and extensive death to specific cells in the spleen, thymus, bone marrow, and intestinal epithelium. This phenomenon of cellular and nuclear disintegration, now defined as apoptosis (*1*) and long known to be an outcome of whole-body irradiation in these mammalian cell types, has been most carefully studied in spleen, thymus, and intestinal epithelia (*2*).

In contrast to extensive morphological studies, biochemical pathways that govern the differential survival or repair of normal cells exposed to ionizing radiation in vivo are only beginning to be defined. The discovery that ionizing radiation-induced growth arrest or apoptotic pathway is dependent on the tumor suppressor protein p53 (*3*), prompted further examination of the response of the p53 pathway to ionizing radiation injury in vivo and provided the most accurate polypeptide marker known whose modification can provide a direct readout of ionizing radiation injury in vivo.

Current methods that detect perturbation of the ionizing radiation-dependent p53 pathway include:

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1. Increases in p53 protein levels.
2. Increase in expression of gene products under transcriptional control of p53 protein.
3. Induction of apoptosis in some cell types.

Certain cells of spleen, thymus, bone marrow, and intestinal epithelia are sensitive to ionizing radiation-dependent apoptosis in a p53-dependent manner (4–8). However, most cell types do not suffer any acute morphological defects following radiation injury. Of the organs that do not suffer from radiation-induced death, select cell types activate the transcriptional function of p53, including the lung, kidney (9), and salivary gland duct epithelia (10). In contrast to this variable induction of the p53-dependent apoptotic and transcriptional function in vivo, increases in p53 protein levels after radiation exposure appear to provide a striking readout of radiation injury in vivo. An approximately two- to fourfold increase in p53 protein levels can be observed after radiation damage regardless of organ type, thus providing a relatively unique and sensitive protein marker of radiation injury. This chapter describes sensitive immunochemical and cell-staining methods for quantitating p53 protein in tissues.

2. Materials

2.1. Cell Lysis

1. Lysis buffer: 1% Nonidet P-40 (NP-40), 25 mM HEPES, pH 7.6, 5 mM dithiothreitol (DTT), 50 mM NaF, 0.15 M KCl, 1 mM benzamidine.
2. Liquid nitrogen.
3. Hand-held pestle for 1.5-mL microcentrifuge tubes.
4. Refrigerated (2°C) microcentrifuge.
5. Protein assay kit (Bio-Rad).

2.2. ELISA

1. Antimouse p53 monoclonal antibody (MAb) PAb248 or PAb246 (Oncogene Sciences), rabbit antimouse p53 polyclonal CM5 antibody (Novocastra, Newcastle upon tyne, UK), goat antirabbit horseradish peroxidase (GAR-HRP) IgG antibody.
2. 96-Well ELISA plates.
3. 0.1 M sodium borate buffer, pH 9.0.
4. PTMB buffer: phosphate-buffered saline (PBS) containing 0.1% Tween-20, 3% milk powder, and 5% bovine serum albumin (BSA).
5. PT buffer: PBS containing 0.1% Tween-20.
6. Capture buffer: 20% glycerol, 25 mM HEPES, pH 7.6, 5 mM DTT, 0.15 M KCl, 0.1% Tween-20, 5% BSA, 3% milk powder, 1 mM benzamidine, and 50 mM NaF.
7. ECL kit (Amersham).
8. Chemiluminescence-ELISA plate reader.

2.3. Gel-Shift Assay

1. DNA binding buffer: 20% glycerol, 25 mM HEPES, pH 7.6, 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 1 mg/mL BSA, 0.1% Triton X-100.
2. T4 Polynucleotide kinase end-labeled oligonucleotides containing the 20-mer p53 consensus DNA binding site (PG): AGACATGCCTAGACATGCCT.
3. Competitor DNA: pBluescript (Stratagene), poly dI/dC, or salmon sperm DNA.
4. Antimouse p53 antibodies PAb421 and PAb248 (Oncogene Sciences).
5. Polyacrylamide gel electrophoresis equipment.

2.4. Immunohistochemical Staining

1. Buffered formalin.
2. Paraffin wax.
3. Poly-L-lysine coated slides.
4. Hydrogen peroxide solution: 1% hydrogen peroxide in PBS.
5. 10 mM Citrate buffer, pH 6.0.
6. 5% Normal swine serum (NSS).
7. Anti-p53 CM5 antibody.
8. Swine antirabbit HRP antibody (DAKO).
9. 3,3' Diaminobenzidine (DAB) solution: Add 1 g of DAB/50 mL of PBS and warm to 50–60°C to dissolve.
10. Developing solution: Add 200 μ L of DAB solution to 40 mL of PBS, then add 30 μ L of H₂O₂, and mix. This solution must be prepared immediately before use.
11. Hematoxylin (BDH).
12. HistoClear (National Diagnostics, Atlanta, GA).
13. DPX mounting medium (BDH).

3. Methods

3.1. Cell Lysis

1. Retrieve tissues from nonirradiated or irradiated animals, and freeze immediately in liquid nitrogen. Store at –80°C.
2. Lyse tissues in a frozen state by homogenization in a 1.5-mL microfuge tube using a handheld pestle in approx 3 volumes of lysis buffer. Lysis from frozen cells is important to minimize phosphatase and protease liberation prior to buffer addition. Following a 20-min incubation on ice, sediment the lysate in a refrigerated microcentrifuge (2°C) at 12,000g for 10 min.
3. Recover the soluble supernatant and, prior to storage at –80°C, determine the protein concentration by the method of Bradford (e.g., using a Bio-Rad protein assay kit).

3.2. Nondenaturing ELISA (see Note 1)

1. Coat individual wells from a 96-well ELISA plate with PAb248 or PAb246 antibody in 50 μ L of sodium borate buffer overnight at 2°C. The antibody concentration should be 1 μ g/mL or 50 ng of MAb/well (see Note 2).

2. Remove the antibody solution by aspiration and block the remaining reactive surfaces by the addition of 200 μL of PTMB buffer.
3. Following incubation at room temperature for 1 h, remove the PTMB buffer and wash the wells four times with PT buffer to remove any remaining unbound MAb.
4. The p53 protein-capture reactions are performed as follows. Prepare an increasing twofold dilution series of the concentrated cell lysate initially starting from 500 μg of protein and continuing to 5 μg of protein in a final volume of 50 μL . Add 50 μL of capture buffer to each dilution, and incubate for 4 h at 0°C.
5. Wash the wells three times with PT buffer to remove the cell lysate, and add a 1:1000 dilution of CM5 antibody in 50 μL of PTMB buffer. Continue the incubations for 1 h at room temperature.
6. Wash the wells three times with PT buffer to remove unbound primary antibody, and add a 1:1000 dilution of GAR-HRP secondary antibody in 50 μL of PTMB buffer. Continue the incubations for 1 h at room temperature.
7. Wash the wells three times with PT buffer to remove any unbound secondary antibody.
8. Initiate the chemiluminescence assay by adding 50 μL of ECL solution to each well, and place the microtiter plate within the cavity of an opaque ELISA plate reader. The peroxidase enzyme activity can be quantitated up to 1 h following the addition of the ECL substrate. ECL-based quantitation is essential for the detection of the low levels of p53 protein extracted from tissues.

3.3. Sequence-Specific DNA Binding Gel-Mobility Shift Assay

The biochemical activity of p53 most tightly linked to its biological function involves its ability to bind to a specific DNA sequence and function as a transcription factor. Increases in p53 protein DNA binding activity in response to DNA damage can be quantitated using in vitro sequence-specific DNA binding assays in which p53 protein forms a complex with its 20-bp consensus DNA element. p53 protein activity is difficult to detect in organs owing to its relatively low levels. As such, in vitro DNA binding assays contain the antibodies PAb421 and PAb248 to focus and supershift the p53–DNA complexes to a very large molecular weight that is well separated from contaminating nonspecific proteins that bind to the radiolabeled oligonucleotides in the cell lysate.

1. Set up the following 20 μL reaction for each cell lysate dilution (*see Note 3*):
 - a. DNA binding buffer 10 μL
 - b. Radiolabeled PG DNA 1 ng
 - c. Competitor DNA Titrate (500 ng to 2 μg)
 - d. Anti-p53 antibody 50 ng
 - e. Cell-lysate dilution Titrate (50–0.1 μg)
- Incubate at 0°C for 20 min.

2. Load the reaction products onto a 4% polyacrylamide gel containing 0.33X TBE and 0.1% Triton X-100, which has undergone pre-electrophoresis at 100 V for 15 min at 3°C. Continue electrophoresis at 200 V for 30–90 min at 3°C.
3. Dry the gel and expose to X-ray film.

3.4. Immunohistochemical Staining of Tissue

In contrast to immunochemistry, immunohistochemistry allows the detection of cell-type-specific induction of p53 in response to DNA damage that is not achieved by other immunochemical methods. This method is of particular use in paraffin sections, since both treated and untreated tissues can be mounted on the same slide to act as an internal control.

1. Retrieve tissues, fix in buffered formalin overnight, and embed in paraffin wax according to standard methods (**10**). Cut 4- μ m sections, and mount on poly-L-lysine-coated slides. Allow to dry overnight at 37°C. Place irradiated and unirradiated tissues side by side on the same slide.
2. Dewax and rehydrate the slides according to the following steps: wash twice in HistoClear for 10 min each. Wash twice in absolute alcohol for 5 min each. Wash twice in methylated spirits for 5 min each. Wash twice in PBS for 5 min each.
3. Incubate the slides in 1% hydrogen peroxide solution on a shaker for 20 min to block endogenous peroxidase activity.
4. Wash the slides twice in PBS for 5 min each.
5. Formalin preservation causes masking of antigens from antibody recognition. To retrieve the antigens, the slides are boiled. Immerse in warm citrate buffer and boil in a microwave at full power (750 W) for 10–20 min. The time required for boiling may vary between microwaves. Therefore, a range of times should be tested to achieve the best results. Do not allow the slides to dry. Cool the slides under slow-running tap water.
6. Wash the slides twice in PBS for 5 min each.
7. Block the slides by covering them with 5% normal swine serum for 30 min in a humidifying incubator.
8. Tap off excess serum. Add CM5 antibody (diluted 1:5000–1:10,000) to cover the sections completely. Incubate overnight at 4°C.
9. Wash the slides three times with PBS for 5 min each with shaking.
10. Incubate with swine antirabbit HRP-conjugated antibody (diluted 1:100) for 1 h in the incubator.
11. Wash the slides as in **step 9**.
12. Prepare the developing solution, pour it over the slides, and incubate for 5–10 min.
13. Wash the slides three times in tap water, and counterstain with hematoxylin.
14. Dehydrate the slides by passing them back through the alcohol solutions: twice in methylated spirits for 5 min each; twice in absolute alcohol for 5 min each; twice in HistoClear for 5 min each. Mount with DPX resin under a cover slip and view using a light microscope.

An example of this staining showing the difference in p53 levels between irradiated and unirradiated tissues can be found in ref. (10).

4. Notes

1. Alternative procedure: p53 immunoprecipitation protocol. Owing to the low abundance of p53 protein in most organs, a combined immunoprecipitation with MAbs PAb248 and PAb246 and immunoblotting with the polyclonal antibody CM-5 is required to document changes in levels of p53 protein (10).
 - a. Incubate 500 μ g of cell lysate by rotating in a mixer for 2 h at 4°C in 200 μ L of IP buffer (PBS containing 0.1% Tween-20, 5 mM DTT, 50 mM NaF, 0.15 M KCl, and 1 mM benzamidine) containing 1 μ g of PAb248 or PAb246 antibodies covalently crosslinked to 20 μ L of protein G beads (Pharmacia). Crosslinking of IgG to protein G beads is required to reduce the background from IgG heavy chain observed when performing immunoblots with polyclonal antibody CM5. Dimethyl pimilimide is used to cross link the IgG to protein G beads chemically according to previously published methods (11). Briefly, antibody is bound to protein G beads (20 μ g of MAb/20 μ L of beads), chemically crosslinked, and washed in appropriate buffers.
 - b. Wash the beads three times with 500 μ L of IP buffer. Elute the bound protein with SDS sample buffer (4% SDS; 20 mM Tris-HCl, pH 6.8; 10% glycerol; and 0.1 M DTT). Load the solubilized samples onto a 10% SDS-polyacrylamide gel. Following electrophoresis, transfer the protein to a nitrocellulose membrane (Amersham) in transfer buffer (20% methanol; 90 mM glycine; 12 mM Tris-OH) at 20 mA for 18 h or 250 mA for 2 h with cooling.
 - c. Block the protein binding sites on the nitrocellulose membrane by incubation in PTM buffer (PBS containing 0.1% Tween-20 and 5% nonfat milk powder) for 1 h at room temperature on a rotating platform.
 - d. Incubate the blot with CM5 antibody (diluted 1:1000) in PTM buffer.
 - e. Wash the blot three times in PT buffer, and incubate with GAR-HRP (diluted 1:1000) or rabbit antimouse antibody linked to HRP for 1 h at room temperature in PTM buffer. Wash the blots three times with PT buffer. Detect peroxidase activity using an ECL kit (Amersham). Approximately 1 s to 5 min are required to detect p53 protein in lysates, depending on the p53 concentration.
2. Although most methods employ the use of MAbs at 30 μ g/mL, thus precluding the use of this assay for many laboratories owing to the expense, this amount of antibody is an overestimation of the amount required.
3. When assaying p53 protein in whole-cell lysates, it is important to titrate both the lysate and different types of competitor DNA, and optimize the assay using cocktails of different DNAs. This is owing to the fact that different cell types have different levels of p53 protein, DNA binding proteins, and nucleases that can potentially compete with p53 in binding to the radiolabeled DNA.

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Activation of p53 Protein Function in Response to Cellular Irradiation

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1. Introduction

p53 protein is a key regulatory component of a stress-inducible cell-cycle checkpoint pathway in mammalian cells, which can promote either cell-growth arrest or apoptosis, depending on the type of cell and damaging agent utilized. Environmental insults that can activate the p53 pathway are quite distinct, and include ionizing and nonionizing radiation (*1–5*), antimetabolites which inhibit ribonucleotide biosynthesis (*6*), inhibitors of spindle formation (*7*), microtubule-affecting drugs (*8*), factors inducing differentiation (*9*), signaling pathways activated in transformed cells during anchorage-independent growth (*10*), heat shock (*11*), and hypoxia (*12*).

Since p53 protein is proving to be a classic marker of many distinct types of cellular injury, as mentioned above, there is increasing interest in using simplified methods to detect activation of the p53 pathway. The activation of p53 protein function by DNA damage can be grouped historically into three categories:

1. Induction of p53 protein levels (*13,14*).
2. Induction of sequence-specific DNA binding (*15*) and transcriptional activity (*14*).
3. Induction of a growth arrest in proliferating cells in culture (*13,14*).

This chapter describes current methods that can be used to quantitate p53 protein induction or activity in response to cellular radiation injury. Each method has its own advantages; the ELISA can be used to quantitate native, folded p53 tetramers, and provides an accurate analysis of the wild-type or mutant conformation of p53; the DNA binding assay is useful to quantitate the amount of latent or activated p53 protein; and the cell-staining assay can give

an indication of the cellular localization of p53 and the variability in expression in a population of cells.

2. Materials

2.1. Cell Lysis

1. Phosphate-buffered saline (PBS), pH 7.4.
2. Liquid nitrogen.
3. Lysis buffer: 1% Nonidet P-40 (NP-40) or 1% IGEPAL, 25 mM HEPES, pH 7.6, 5 mM dithiothreitol (DTT), 50 mM NaF, 0.5 M KCl, 1 mM benzamidine.
4. Protein assay kit (Bio-Rad).

2.2. ELISA

1. Microtiter plates (Falcon, 3912).
2. Anti-p53 monoclonal antibody (MAb) DO-1 (for human p53) or PAb248 or PAb246 (for mouse p53) (Oncogene Sciences).
3. Anti-p53 polyclonal antibody CM1 (human) or CM5 (mouse) (Novocastra).
4. Goat antirabbit horse radish peroxidase-conjugated antibody (GAR-HRP) (DAKO) or donkey antirabbit HRP.
5. 0.1 M Sodium borate, pH 9.0 (Sigma).
6. PTB buffer: PBS containing 0.1% Tween-20, 5% bovine serum albumin (BSA) (Sigma).
7. PT buffer: PBS containing 0.1% Tween-20.
8. Capture buffer: 20% glycerol, 25 mM HEPES, pH 7.6, 5 mM DTT, 0.15 M KCl, 0.1 % Tween-20, 5% benzamidine, 50 mM NaF.
9. ECL protein detection kit (Amersham).

2.3. DNA Binding Activity Assay

1. DNA binding buffer: 20% glycerol, 25 mM HEPES, pH 7.6, 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 1 mg/mL BSA, and 0.1% Triton X-100.
2. Competitor DNA: pBluescript II (Stratagene), poly dI/dC, a nonspecific oligonucleotide, or salmon sperm DNA (Sigma).
3. Radiolabeled PG oligonucleotide: AGACATGCCTAGACATGCCT
4. Anti-p53 MAb DO-1, PAb421.
5. Polyacrylamide gel electrophoresis equipment.

2.4. Immunostaining of Cells in Culture

1. Thermanox coverslips (Nunc).
2. Tissue-culture dishes, 35–90 cm².
3. PBS, pH 7.4.
4. PBS containing 0.6% BSA.
5. Anti-p53 MABs: DO-1 or PAb1801 (Oncogene Sciences) to detect human p53. PAb248 (Oncogene Sciences) to detect mouse p53 (*see Note 1*). CM5 rabbit polyclonal antibody can also be used to detect mouse p53.

6. Horse radish peroxidase-conjugated rabbit antimouse IgG (DAKO).
7. DAB solution: 2.5 mM diaminobenzidine (DAB) (Sigma), 0.1% H₂O₂, made fresh.
8. DPX mounting medium (BDH).

3. Methods

Both ionizing and nonionizing radiation can activate the p53 pathway, although the kinetics of p53 protein induction, activation of p53 transcriptional activity, and induction of a growth arrest are quantitatively distinct between the two types of radiation. In general, ionizing radiation induces a relatively rapid and striking p53-dependent growth arrest in normal fibroblasts with very weak increases in p53 protein levels (twofold). In contrast, nonionizing radiation induces a growth arrest in which p53 protein levels can slowly and dramatically increase, but protein levels can be uncoupled from transcriptional activity (9,16). As such, when examining radiation-dependent perturbation of the p53 pathway using the assays described below, time-courses and radiation dose curves should be employed to characterize accurately the p53 response.

3.1. Cell Lysis

1. Wash irradiated or nonirradiated cells in a tissue-culture dish three times with PBS and scrape with a rubber policeman into 1.5 mL of PBS. It is important that the cells are washed thoroughly to remove serum proteins that will interfere with protein determinations following cellular lysis.
2. Transfer the cell suspension to a 1.5-mL microcentrifuge tube, and centrifuge in a refrigerated microcentrifuge (2°C) at 1000 rpm (~80g) for 5 min. Freeze the cell pellet in liquid nitrogen for subsequent lysis.
3. Add approx 1 vol of lysis buffer to the frozen cell pellet, and resuspend the cells by gently repipeting. Such concentrated lysates aid in capturing p53 protein when its concentration is very low. (If the lysates are to be used only for immunoblotting, use 2 vol of lysis buffer.) (See Note 2.)
4. Following a 20-min incubation on ice, sediment the suspension in a refrigerated microcentrifuge at 12,000g for 10 min. Recover the soluble supernatant and determine the protein concentration by the method of Bradford (e.g., using a Bio-Rad protein assay kit) prior to storage at -80°C.

3.2. Nondenaturing ELISA (see Note 2)

1. Coat individual wells from a 96-well ELISA plate with 50 ng of appropriate MAb (DO-1 for human p53, PAb248 or PAb246 for murine p53) in 50 µL of sodium borate buffer overnight at 2°C (see Note 3).
2. Remove the antibody solution by aspiration and block the remaining reactive surfaces by adding 200 µL of PTB buffer.
3. Following incubation at room temperature for 1 h, remove the PTB buffer and wash the wells four times with PT buffer to remove any unbound MAb.

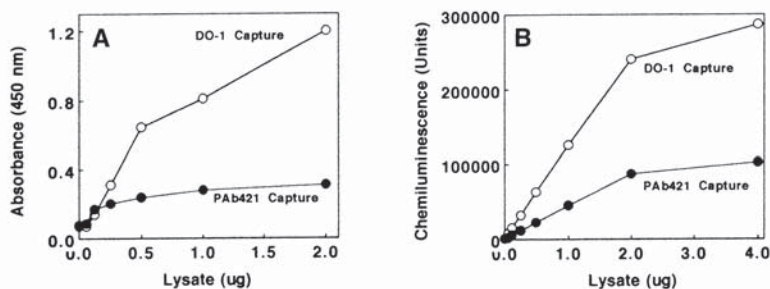


Fig. 1. Representative capture ELISA from human tumor cell lysates (T47D) with human p53 protein, developed using monoclonal DO-1 as a capture antibody (most sensitive) or the MAb PAB421 as a capture antibody (least sensitive) and polyclonal antibody CM1 as the detection antibody. (A) and (B) compare TMB- and ECL-based quantitation methods (*see Note 4*).

4. The p53 protein-capture reactions are performed as follows. Starting with 25–50 μ g of protein, prepare in capture buffer an increasing twofold dilution series of the concentrated cell lysate. Add 50 μ L of each dilution to the microtiter plate and incubate for 2–4 h at 2°C.
5. Wash the wells three times with PT buffer to remove the cell lysate, and add 50 μ L of a 1:1000 dilution of rabbit polyclonal antibody in 50 μ L of PTB buffer (CM1 or CM5, specific for human or mouse p53, respectively). Incubate for 1 h at room temperature.
6. Wash the wells three times with PT buffer to remove the primary antibody, and add 50 μ L of a 1:1000 dilution GAR-HRP secondary antibody. Incubate for 1 h at room temperature.
7. Wash the wells three times with PT buffer to remove any unbound secondary antibody. Quantitate the amount of captured p53 using an ECL chemiluminescence-based assay (*see Note 4*).
8. Initiate the chemiluminescence assay by adding 50 μ L of ECL solution, and place the microtiter plate within the cavity of an opaque ELISA plate reader. The peroxidase enzyme activity can be quantitated up to 1 h following ECL-based substrate addition.

A representative capture ELISA is shown in **Fig. 1**.

3.3. Sequence-Specific DNA Binding Activity In Vitro

Increases in p53 protein activity in response to DNA damage can be quantitated using in vitro sequence-specific DNA binding assays in which p53 protein forms a complex with its 20-bp consensus DNA element (17,18). p53 protein induced by cellular irradiation is often produced in at least two distinct forms with respect to sequence-specific DNA binding: one in a latent state and

another in an active form (**15**). As such, in vitro DNA binding assays are complicated by the fact that p53 protein levels do not often correlate with sequence-specific DNA binding activity. Nevertheless, the two DNA binding forms of p53 produced can be quantified as described below.

Activation of latent forms of p53 can be accomplished in vitro by the use of site-specific protein kinases (**19**). A MAb (PAb421) whose epitope overlaps the C-terminal regulatory phosphorylation sites can mimic the effects of kinases and also activate p53 protein in vitro (**17**). As such, the antibody PAb421 has served as a useful reagent to quantitate the amount of latent p53 produced by cells. In addition, the ratio of activated to latent p53 protein in cell lysates can be quantitated using antibody-assisted DNA binding assays; the addition of DO-1 antibody to a DNA binding reaction measures activated p53, and the addition of PAb421 measures the amount of both active and latent p53.

1. Prepare cell lysates as described in **Subheading 3.1.**, including a twofold dilution series of lysate.
2. Set up the following 20- μ L reactions (*see Note 5*):

DNA binding buffer	10 μ L
Radiolabeled DNA (PG)	1–5 ng
Competitor DNA	1 μ g
DO-1 or PAb421 antibody	100 ng
Cell lysate	Dilutions
3. Following incubation at 0°C for 20 min, load onto a 4% polyacrylamide gel containing 0.33X TBE and 0.1% Triton X-100, which has undergone pre-electrophoresis at 100 V for 15 min at 3°C. Electrophorese at 200 V for 30–90 min at 3°C. Dry the gel and expose to X-ray film.

3.4. Immunohistochemical Staining of Cells in Culture (see Note 6)

Although immunochemical methods provide straightforward and quantitative approaches to detect changes in p53 protein levels, immunohistochemical methods give an added advantage of being able to detect nuclear localization of p53 rapidly (**14**).

1. Grow cells on Thermanox cover slips in tissue-culture dishes.
2. Wash twice with PBS and immediately immerse in cold (–20°C) 50% methanol/50% acetone. Fix for 10 min at –20°C.
3. Allow the cover slips to air-dry. They can be used immediately or stored for several weeks at –20°C in the presence of desiccant.
4. Rehydrate the cells by briefly immersing in PBS. At no point subsequent to this should the cells be allowed to dry out.
5. Place the cover slips on a piece of parafilm and cover with 30 μ L of primary MAb (DO-1 or PAb1801 for human p53, PAb248 or CM5 rabbit polyclonal for mouse p53, *see Note 1*) diluted in PBS containing 0.6% BSA. The antibody concentra-

tion should be titrated for optimal results; 1 $\mu\text{g/mL}$ is suitable for most antibodies.

6. Incubate for 1 h at room temperature in a humidified incubator, and then wash three times with PBS.
7. Cover the slides with peroxidase-conjugated rabbit antimouse IgG (or goat antirabbit HRP for CM5), diluted 1:1000 in PBS containing 0.6% BSA. Incubate for a further 1 h.
8. Wash three times in PBS, then add freshly prepared DAB solution, and incubate for 3–5 min, during which time a brown precipitate should form at the site of antibody localization. Wash in PBS.
9. Dehydrate the cells by briefly immersing the cover slips in 100% ethanol. Dip them in xylene, and mount on microscope slides using DPX mounting medium.

4. Notes

1. The MAb PAb421 can detect both human and murine p53, but the epitope is blocked by posttranslational modification (17) and, therefore, is not necessarily a reliable antibody to detect changes in p53 protein levels.
2. Alternative protocol: p53 immunoblot:
 - a. Harvest the cells as described in **steps 1 and 2 of Subheading 3.1.**
 - b. Add approx 2 vol of lysis buffer (1% NP-40 or 1% IGEPAL, 25 mM HEPES, pH 7.6, 5 mM DTT, 50 mM NaF, 0.5 M KCl, 1 mM benzamidine) to the frozen cell pellet, and resuspend by gently repipeting. Lysis from frozen cells is important to minimize phosphatase and protease liberation prior to buffer addition.
 - c. Following a 20-min incubation on ice, pellet the suspension in a refrigerated microcentrifuge (2°C) at 12,000g for 10 min.
 - d. Recover the soluble supernatant and perform a protein determination prior to storage (*see step 4, Subheading 3.1.*).
 - e. Apply 5–50 μg of lysate to a 10% SDS-polyacrylamide gel. Following electrophoresis, transfer the protein to a nitrocellulose membrane (Amersham) in transfer buffer (20% methanol, 90 mM glycine, 12 mM Tris base) at 20 mA for 18 h, or 250 mA for 2 h with cooling.
 - f. Block the membrane by incubation in PTM buffer (PBS containing 0.1% Tween-20 and 5% nonfat milk powder) for 1 h at room temperature on a rotating platform.
 - g. Incubate the blot with anti-p53 MAb or polyclonal antibody in PTM buffer. The concentration of antibody should be 1 $\mu\text{g/mL}$ for pure MAbs or diluted 1:2000 for high-titer polyclonal antibodies. Overnight incubation of the membrane at 2°C increases the sensitivity of the immunoblot assay. The MAb DO-1 is the preferred and most sensitive antibody for detecting human p53 and the antibody PAb248 or BP19.1 (Novocastra) is preferred for murine p53.
 - h. Wash the blot three times in PT buffer and incubate with a 1:100 dilution of GAR-HRP or rabbit antimouse antibody linked to HRP for 1 h at room temperature in PTM buffer. Wash the blot three times with PT buffer. Detect

peroxidase activity using ECL solution (Amersham). Approximately 1 s to 5 min are required to detect p53 from lysates.

3. Although most methods employ the use of MAbs at 30 $\mu\text{g/mL}$, thus precluding the use of this assay for many laboratories owing to the expense, this amount of antibody is an overestimation of the amount required. The antibody concentration can be as low as 0.1 $\mu\text{g/mL}$ or 5 ng of antibody/well. The monoclonal DO-1 is the most sensitive for human p53, and the MAbs PAb248 or Pab246 are best for detecting murine p53.
4. The amount of p53 captured can also be quantitated using a TMB-based colorimetric assay (**Fig. 1A**). The assay is initiated by adding 50 μL of TMB Assay Solution (Sigma) and incubating at room temperature for 5–10 min. The reactions are terminated by adding 50 μL of 0.1 *N* sulfuric acid, and the optical density is determined at 450 nm using an ELISA plate reader. The distinct advantage of ECL-based quantitation is that the assay is linear over a 60- to 200-fold range. In contrast, the TMB-based assay is often linear over a two- to three-fold range, and it is usually 10–20 times less sensitive. Using cellular lysates, the chemiluminescent assay is obviously an advantage, but accurate readings can be obtained using the TMB assay.
5. When assaying p53 protein in whole-cell lysates, it is important to titrate different types of competitor DNA and optimize the assay using cocktails of different DNAs. This is because different cell types have different levels of DNA binding proteins and nucleases that can potentially compete with p53 protein in binding to the radiolabeled DNA.
6. This method is suitable for the detection of nuclear p53 expressed following exposure to genotoxic agents, or constitutively present at high levels in tumor cell lines with mutant p53. If required, the sensitivity may be increased by the use of a commercial peroxidase amplification system, such as the Novostain super ABC kit from Novocastra. Alternatively, formaldehyde-based fixation procedures (20) are required for the optimal detection of cytoplasmic p53 protein, which may be present in some tumor cells.

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Selective Extraction of Fragmented DNA from Apoptotic Cells for Analysis by Gel Electrophoresis and Identification of Apoptotic Cells by Flow Cytometry

Zbigniew Darzynkiewicz and Gloria Juan

1. Introduction

A characteristic feature of apoptosis is activation of an endonuclease(s), which has preference for internucleosomal DNA (reviewed in *1-5*). As a result, the products of DNA cleavage during apoptosis are discontinuous DNA sections of mono- and oligonucleosome size, which generate a typical “ladder” pattern during gel electrophoresis. This electrophoretic pattern is considered to be a hallmark of apoptosis (*1-5*). Although exceptions to this pattern have been observed (*6-10*), analysis of DNA size on agarose gels is a widely used procedure to reveal apoptosis. Generally, DNA for gel electrophoresis is extracted with phenol or recovered using other standard isolation procedures.

A simple, rapid, and selective procedure for extracting fragmented DNA from apoptotic cells has been developed by us (*11*). In this method, the cells are initially prefixed in 70% ethanol and subsequently, after centrifugation, resuspended in a small volume of high-molarity phosphate-citrate buffer. Because fixation in ethanol is inadequate to retain the fragmented DNA within the cell, this DNA is selectively extracted into the buffer, whereas high mol-wt DNA and DNA attached to the nuclear matrix resist extraction. DNA extraction appears to be facilitated at this slightly alkaline pH and under conditions of relatively high ionic strength, where the electrostatic interactions between DNA and cellular proteins (which otherwise restrict elution of DNA from the cells) are weakened. The DNA extract is incubated with RNase A, then with proteinase K, and after a brief additional incubation, the extract is subjected to

electrophoresis. Apoptotic cells from which fragmented DNA has been removed still contain high-mol-wt DNA, which following staining with any DNA fluorochrome can be distinguished by flow cytometry as cells with a fractional DNA content. On DNA content frequency histograms, they are represented by the “sub-G₁ peak” (5,12,13).

The procedure of DNA extraction is simple, rapid, and does not employ toxic reagents (e.g., phenol, chloroform), which are used in alternative methods. DNA to be examined by gel electrophoresis is extracted from the very same cell population that is subjected to measurements by flow cytometry. The latter measurement allows one to estimate DNA ploidy, the cell-cycle distribution of nonapoptotic cells, and the percentage of apoptotic cells, or other parameters. The method is applicable to clinical samples, which when fixed, can be in ethanol, and then stored and/or safely transported prior to analysis.

2. Materials

2.1. Equipment and Instruments for DNA Gel Electrophoresis

The procedures of DNA isolation and gel electrophoresis do not require any special instrumentation or materials. The following equipment and materials, which can be acquired from general suppliers, such as Fisher Scientific or Cole-Parmer Instrument Co., are required.

1. Eppendorf microcentrifuge tubes, 0.5 mL.
2. 15-mL polypropylene centrifuge tubes.
3. Standard centrifuges.
4. Gel electrophoresis apparatus (horizontal).
5. UV light transilluminator (e.g., Fotodyne UV 300 analytic DNA transilluminator, Fotodyne, New Berlin, WI).
6. Polaroid photographic camera.
7. Polaroid type 667 film (ASA 3000).
8. Filters for photography: Orange filter (Kodak Wratten #23A); transparent UV light blocking filter (Kodak Wratten # 2B).
9. Water bath with shaker.
10. Heating plate.

2.2. Equipment and Instruments for Flow Cytometry

Flow cytometers of different types, offered by several manufacturers, can be used to measure cell fluorescence following staining according to the procedure described below. The manufacturers of the most common flow cytometers are Coulter Corporation (Miami, FL), Becton Dickinson Immunocytometry Systems (San Jose, CA), Cytomation (Fort Collins, CO), and PARTEC (Zurich, Switzerland). The multiparameter Laser Scanning Cytometer (LSC) is available from CompuCyte, Inc. (Cambridge, MA). The

Cytospin centrifuge, which is used in conjunction with LSC, is available from Shandon (Pittsburgh, PA).

The software to deconvolute the DNA content frequency histograms, to analyze the cell-cycle distributions is available from Phoenix Flow Systems (San Diego, CA) and Verity Software House (Topham, MA).

2.3. Reagents

1. 70 % Ethanol. Prior to cell fixation, distribute 10-mL aliquots of 70% ethanol into 15-mL polypropylene centrifuge tubes. Keep them on ice.
2. DNA extraction buffer: Mix 192 mL of 0.2 M Na₂HPO₄ with 8 mL of 0.1 M citric acid (the pH of this buffer is 7.8).
3. RNase A stock solution: Dissolve 2 mg of DNase-free RNase (Sigma, St. Louis, MO) in 1 mL of distilled water. If the RNase is not DNase-free, heat this solution at 100°C for 5 min to inactivate any traces of DNase.
4. Proteinase K stock solution: Dissolve 1 mg of proteinase K (Sigma) in 1 mL of distilled water.
5. Electrophoresis buffer (10X TBE): Dissolve 54 g of Tris base (Sigma) and 27.5 g of boric acid in 980 mL of distilled water; add 20 mL 0.5 M of EDTA (pH 8.0).
6. Agarose gel (0.8%): Dissolve 1.6 g of agarose in 200 mL of hot (boiling) 1X TBE. Pour the solution onto a 15 × 15 cm² plate. Cool to room temperature.
7. Loading buffer: Dissolve 0.25 g of bromophenol blue (Sigma) and 0.25 g of xylene cyanol (Sigma) in 70 mL of distilled water. Add 30 mL of glycerol (Sigma).
8. Ethidium bromide (EB) stock solution: Dissolve 1 mg of EB (Molecular Probes, Eugene, OR) in 1 mL of distilled water. This solution can be stored at 4°C in the dark for months.
9. EB staining solution: For staining gels, add 20 µL of stock solution to 200 mL of TBE. **EB is a suspected carcinogen. Wear gloves and observe caution.**
10. DNA mol-wt standards: DNA from 100–1000 bp in length.
11. DNA staining solution for flow cytometric cell analysis: Dissolve 200 µg of propidium iodide (PI) (Molecular Probes) in 10 mL of PBS. Add 2 mg of DNase-free RNase A (*see item 3*). Prepare fresh solutions before each use.

3. Methods

3.1. DNA Gel Electrophoresis (*see Notes 1 and 2*)

1. Collect cultured cells, and suspend 1–5 × 10⁶ cells in 1 mL of PBS (*see Note 3*).
2. Fix cells in suspension in 70% ethanol by admixing, with a Pasteur pipet, the above cell suspension into 10 mL of 70% ethanol in centrifuge tubes on ice (*see Note 4*).
3. Centrifuge the cells at 300g for 5 min. **Thoroughly** decant the ethanol, leaving only the cell pellet. Add 50 µL of extraction buffer. Cap the tubes and place on the shaker. Shake with moderate intensity for 30 min at room temperature.
4. Centrifuge the cells as in **step 3**. Withdraw all the supernatant, and transfer it to a 0.5-mL Eppendorf tube. Keep the cell pellet for analysis by flow cytometry (*see Subheading 3.2.*).

5. Add 5 μ L of RNase A to the supernatant, mix, and incubate at 37°C for 30 min. Cap the tube to prevent drying.
6. Add 5 μ L of proteinase K, mix, and incubate at 37°C for 30 min. Cap the tube to prevent drying.
7. Add 5 μ L of loading buffer, mix, and transfer the tube contents to a 0.8% agarose horizontal gel (*see Note 5*).
8. Load a sample of DNA standards.
9. Electrophorese the gel at 2 V/cm until the desired resolution is obtained (12–18 h).
10. To visualize the bands, stain the gel with EB staining solution, and transfer to a UV light transilluminator. **UV light is damaging to eyes and skin. Wear protective facewear while observing UV-illuminated gels.**
11. Photograph the gel with Polaroid type 667 film using an orange filter and a transparent UV light blocking filter.

An example of a DNA ladder is shown in **Fig. 1C** (*see Note 6*).

3.2. Detection of Apoptotic Cells by Flow Cytometry

1. Resuspend the cell pellet (from **step 4** of **Subheading 3.1.**) in 1 mL of the DNA staining solution. Keep for 30 min at room temperature in the dark.
2. Set up and adjust the flow cytometer: Excitation should be in blue light. Use 488-nm argon ion laser line, or a B 12 optical filter when the source of illumination is a mercury arc or xenon lamp. Emission of PI is in red wavelengths; a long pass (>600 nm) filter is recommended.
3. Measure cell fluorescence in a flow cytometer. Use pulse width—pulse area signal to discriminate between G₂ cells and cell doublets, and gate out the latter (*see Note 7*).

DNA content frequency histograms are shown in **Fig. 1A,B** (*see Note 6*).

4. Notes

1. In comparison with the conventional methods of DNA extraction the present approach offers the following advantages:
 - a. The cells are fixed in ethanol and therefore may be stored for weeks or months at –20°C. This feature is attractive for use with clinical material (e.g., analysis of apoptosis in blood or bone marrow samples), which can be collected at different times and analyzed later or transported.
 - b. Fixation in ethanol inactivates endogenous enzymes, preventing autolysis after sample collection.
 - c. Ethanol inactivates most viral and bacterial pathogens, making the sample safer to handle.
 - d. The method is rapid and uses less toxic reagents compared to the traditional phenol extraction.
 - e. The ratio of high- to low-mol-wt DNA (i.e., extractable and nonextractable from the ethanol fixed cells) may serve as an index of apoptosis in cell populations. High mol-wt DNA can be extracted and measured by standard biochemical

procedures. It should be noted, however, that the amount of extractable DNA is not only a measure of the frequency of apoptotic cells, but also the degree of DNA fragmentation (progression of apoptosis) in individual cells.

- f. The cells remaining after extraction of the fragmented DNA can be subjected to flow cytometry. The percentage of apoptotic cells, thus, can be estimated in literally the same samples from which the DNA was obtained for gel electrophoresis. In addition, other parameters, such as the cell-cycle distribution of the nonapoptotic cell population, DNA ploidy, cell phenotype, expression of the intracellular antigens, and so forth, all can be measured in the cell population subjected to DNA extraction. This latter feature (f), which makes it possible to apply the complementary approaches of flow cytometry and DNA gel electrophoresis to the same samples, is unique to the present method, since no other technique has been described that offers such a possibility.
2. The presence of apoptotic cells in the suspension of cells extracted with the phosphate-citric acid buffer manifests as a DNA ladder on the gels (**Fig. 1**). The sensitivity of the method, in terms of the minimal number of apoptotic cells that can be detected in the sample, depends primarily on the degree of DNA fragmentation in individual cells. The more advanced the cell is in apoptosis the greater is its fraction of fragmented (extractable) DNA. Because the fragmented, low-mol-wt DNA (i.e., DNA that is responsible for generating the ladder) is selectively extracted from the sample while high-mol-wt remains in the cells, the method appears to be more sensitive compared with the traditional DNA extraction using phenol, where all cellular DNA is extracted. Thus, a relatively small fraction (e.g., 1–2%) of apoptotic cells can be detected if DNA is extracted from a large number ($>10^6$) of cells (**11**).
3. In cultures of cells that adhere to flasks, most apoptotic cells spontaneously detach and float in the medium. Thus, when the medium is discarded and only trypsinized cells are studied, these apoptotic cells also are discarded. To estimate apoptosis in cultures of adhering cells, the detached cells should be pooled with the trypsinized ones.
4. Cells can be stored in fixative at -20°C for several weeks.
5. Transfer smaller (5- to 30- μL) aliquots if a large amount of DNA is expected in the sample (e.g., advanced apoptosis, large fraction of apoptotic cells in the cell population).
6. In some cell types, DNA cleavage may stop after generating 50 to 300-kb fragments without progressing into internucleosomal fragmentation (**6–10**). Such large fragments of DNA cannot be extracted from the ethanol-fixed cells. The method described here, as well other methods of DNA extraction and gel electrophoresis, does not reveal DNA laddering or the presence of distinct sub- G_1 cell populations.
7. Populations of apoptotic cells are discriminated based on reduced DNA content (stainability) in these cells. On the DNA content frequency histograms, apoptotic cells are thus represented by the sub- G_1 peak, which should be separated from the G_1 peak (**Fig. 1**). Depending on the degree of DNA fragmentation and thus

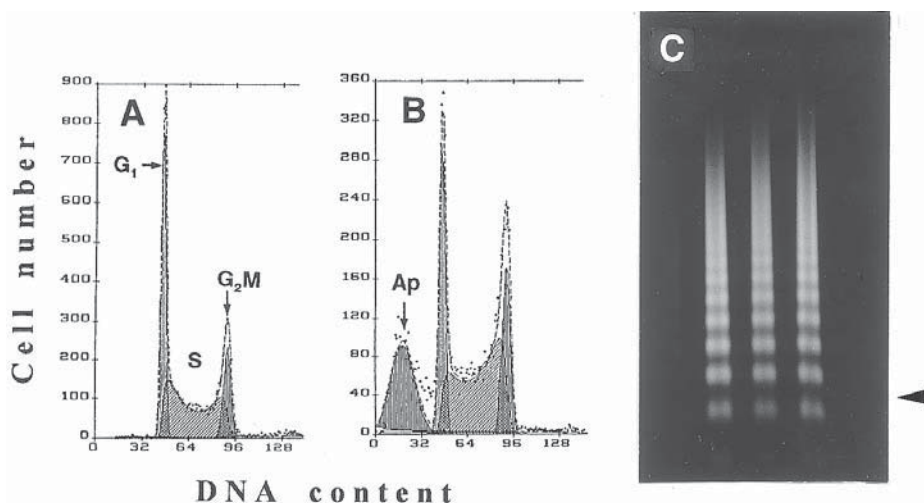


Fig. 1. DNA content frequency histograms of (A) untreated human promyelocytic HL-60 cells and (B) HL-60 cells treated for 4 h with the protein kinase inhibitor genistein (100 $\mu\text{g/mL}$). Following fixation in ethanol, fragmented DNA was extracted from apoptotic cells, the cells were stained with PI as described in the protocol and their DNA was measured by flow cytometry. By deconvolution of the DNA content frequency histograms, it was possible to estimate the proportion of apoptotic cells, represented by the "sub- G_1 " peak (Ap), as well as among the nonapoptotic cell population, the percentage of cells in particular phases of the cycle. The noticeable decrease in the proportion of G_1 cells in the genistein-treated culture (correlated with a decrease in the proportion of cells in S and G_2/M) concomitant with the appearance of the apoptotic cell population, suggests that either the cells residing in G_1 or undergoing transition from G_1 to S during the treatment, were preferentially undergoing apoptosis. (C) A typical agarose gel of DNA extracted by the present method from three HL-60 cultures similarly treated to induce apoptosis shows a distinct "laddering." Note the absence of large-mol-wt DNA at the sites of sample loading, which otherwise is present when total DNA is extracted (e.g., by phenol). The count of the "ladder rungs" indicates that DNA of the size equivalent to 1–15 oligonucleosomes is extracted by this procedure from the ethanol-fixed cells. The arrowhead indicates the position of a 200-bp DNA size marker.

extraction, or the prior loss of cellular DNA via the shedding of apoptotic bodies, the position of the sub- G_1 peak may vary, from near overlap with the G_1 peak to the very low channels. In the case when apoptotic cells are measured at very low fluorescence channels, it is difficult to distinguish them from individual apoptotic bodies, fragments of chromatin, broken nuclei, chromosomes, cell debris, and so forth. The strategies for identification of apoptotic cells in such a situation are discussed in detail elsewhere (5).

Acknowledgments

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Detection of DNA Strand Breakage in the Analysis of Apoptosis and Cell Proliferation by Flow and Laser Scanning Cytometry

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1. Introduction

The presence of DNA strand breaks resulting from the cleavage of nuclear DNA by the apoptosis-associated endonuclease(s) is one of the most characteristic features of apoptotic cells (1,2). A widely used methodology to detect apoptotic cells thus relies on labeling DNA strand breaks *in situ* either with fluorochromes (3,4) or absorption dyes (5–9). The advantage of strand break labeling with fluorochromes is that such cells can be rapidly analyzed by flow cytometry. When cellular DNA content is also measured in these cells, the bivariate analysis of such data provides information about the DNA ploidy and cell-cycle phase specificity of apoptosis (4,10).

The method presented in this chapter can be applied to cells measured by flow cytometry, whereas a simple modification, also presented here, permits the analysis of cells attached to microscope slides. The latter such cells can be analyzed by a new type of instrument, the laser scanning cytometer (LSC). The LSC is a microscope-based cytofluorimeter, which allows one to measure rapidly, with high sensitivity and accuracy, the fluorescence of individual cells (11–13). The instrument combines advantages of both flow and image cytometry. For example, the staining of cells on slides prevents their loss, which otherwise occurs during the repeated centrifugations of samples during preparation for flow cytometry. Another advantage stems from the possibility of spatially localizing particular cells on the slide for their visual inspection or morphometry after the initial measurement of a large population and electronic selection (gating) of the cells of interest. The measured cells, therefore, can be bleached and restained with another set of dyes. This permits the cell attributes

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measured after restaining to be correlated with the attributes measured before, on a cell-by-cell basis (*11–13*).

Cell fixation and permeabilization are essential steps to label DNA strand breaks successfully. Cells are briefly fixed with a crosslinking fixative, such as formaldehyde, and then permeabilized by suspending them in ethanol. By crosslinking low-mol-wt DNA fragments to other cell constituents, formaldehyde prevents extraction of the fragmented DNA, which otherwise occurs during the repeated centrifugations and rinses required by this procedure. The 3'-OH termini of the fragmented DNA serve as primers and become labeled with 5-bromo-2'-deoxyuridine (BrdU) in a reaction catalyzed by exogenous terminal deoxynucleotidyl transferase (TdT) and BrdUTP (*14*). The incorporated BrdU is immunocytochemically detected by anti-BrdU antibody conjugated to fluorescein isothiocyanate (FITC) (*14,15*). This reagent is widely used in studies of cell proliferation to detect BrdU incorporated during DNA replication (*16*). The overall cost of reagents is markedly lower and the sensitivity of DNA strand break detection is higher when BrdUTP is used as a marker, compared to labeling with biotin-, digoxigenin-, or directly fluorochrome-tagged deoxynucleotides (**Fig. 1**) (*14*). However, because certain applications may require the use of multiple fluorochromes, alternative procedures utilizing the latter three reagents are also described in this chapter.

The DNA strand break labeling methodology also can be used for detecting the incorporation of halogenated DNA precursors. In this application, termed “strand breaks induced by photolysis” (SBIP), DNA strand breaks are photolytically generated by UV light in cells that have incorporated BrdU or 5-iodo-2'-deoxyuridine (IdU) which sensitize DNA to UV light (*14,15*). DNA breaks resulting from UV photolysis, which in this case are markers of precursor incorporation (i.e., DNA replication), are subsequently fluorochrome labeled in the same way as described for labeling apoptotic DNA breaks.

2. Materials

2.1. DNA Strand Break Labeling

1. Phosphate-buffered saline (PBS), pH 7.4.
2. 1% Formaldehyde (methanol-free, ultrapure) (Polysciences, Warrington, PA) in PBS, pH 7.4.
3. 70% Ethanol.
4. TdT (Boehringer Mannheim, Indianapolis, IN). TdT 5X reaction buffer: 1 M potassium (or sodium) cacodylate, 125 mM HCl, pH 6.6, 1.25 mg/mL bovine serum albumin (BSA, Sigma, St. Louis, MO). This 5X reaction buffer can be purchased from Boehringer Mannheim.
5. 5-Bromo-2'-deoxyuridine-5'-triphosphate (BrdUTP) stock solution (50 μ L): 2 mM BrdUTP (Sigma) in 50 mM Tris-HCl, pH 7.5.

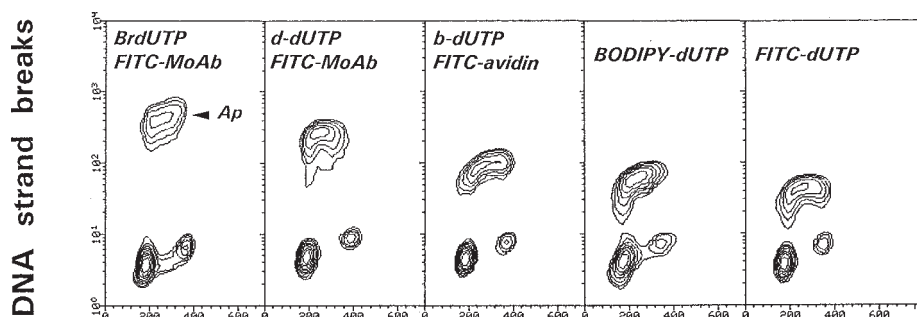


Fig. 1. Detection of apoptotic cells by flow cytometry using different methods of DNA strand break labeling. Bivariate distributions represented by isometric contour maps (DNA content vs DNA strand break labeling) of HL-60 cells incubated for 3 h with $0.15 \mu\text{M}$ camptothecin, which preferentially induces apoptosis (Ap) of DNA replicating cells (4). The first three panels to the left represent indirect labeling of DNA strand breaks, utilizing either BrdUTP, digoxigenin-conjugated dUTP (d-dUTP), or biotinylated dUTP (b-dUTP). The two right panels show cell distributions following a direct, single-step DNA strand break labeling, either with BODIPY- or FITC- conjugated dUTP. Note the exponential scale of the ordinate. As is evident, the greatest difference is achieved following DNA strand break labeling with BrdUTP (14).

6. 10 mM CoCl_2 (Boehringer Mannheim).
7. Rinsing buffer: 0.1% Triton X-100 and 5 mg/mL BSA dissolved in PBS.
8. FITC-conjugated anti-BrdU monoclonal antibody (MAb): Dissolve $0.3 \mu\text{g}$ of FITC-conjugated anti-BrdU MAb (Becton Dickinson, Immunocytometry Systems, San Jose, CA) in $100 \mu\text{L}$ of PBS containing 0.3% Triton X-100 (Sigma) and 1% BSA.
9. Propidium iodide (PI) staining buffer: $5 \mu\text{g/mL}$ PI (Molecular Probes, Eugene, OR), $100 \mu\text{g/mL}$ RNase A (DNase-free) (Sigma) in PBS.
10. Cytospin centrifuge (Shandon, Pittsburgh, PA): for use in conjunction with laser scanning cytometry.

Two kits for labeling DNA strand breaks have been tested by us. APO-BRDU (Phoenix Flow Systems, San Diego, CA) uses a BrdUTP–TdT methodology similar to that described in this chapter. ApopTag (ONCOR, Inc., Gaithersburg, MD) can be used for incorporating digoxigenin-dUTP. A plethora of other kits for DNA strand break labeling is available from different vendors.

2.2. Additional Reagents and Equipment for SBIP

1. BrdU (Sigma).
2. $60 \times 15 \text{ mm}$ polystyrene Petri dishes (Corning, Corning, NY).
3. UV light illumination source: Fotodyne UV 300 analytic DNA transilluminator containing four 15-W bulbs (Fotodyne Inc., New Berlin, WI).

4. UV light photometer: UVX-25 Sensor (UVP, Upland, CA).

2.3. Instrumentation

Flow cytometers of different types, offered by several manufacturers, can be used to measure cell fluorescence following staining according to the procedures described below. The manufacturers of the most common flow cytometers are Coulter Corporation (Miami, FL), Becton Dickinson Immunocytometry Systems (San Jose, CA), Cytomation (Fort Collins, CO) and PARTEC (Zurich, Switzerland). The multiparameter LSC is available from CompuCyte (Cambridge, MA).

The software to deconvolute the DNA content frequency histograms to analyze the cell cycle distributions is available from Phoenix Flow Systems (San Diego, CA) and Verity Software House (Topham, MA).

3. Methods

3.1. Detection of Apoptotic Cells by DNA Strand Break Labeling (see Note 1)

3.1.1. DNA Strand Break Labeling with BrdUTP for Analysis by Flow Cytometry

1. Suspend $1\text{--}2 \times 10^6$ cells in 0.5 mL of PBS. With a Pasteur pipet, transfer this suspension to a 6 mL polypropylene tube (see Note 2) containing 4.5 mL of ice-cold 1% formaldehyde (see Note 3). Incubate on ice for 15 min.
2. Centrifuge at 300g for 5 min, and resuspend the cell pellet in 5 mL of PBS. Centrifuge again, and resuspend the cells in 0.5 mL of PBS. With a Pasteur pipet, transfer the suspension to a tube containing 4.5 mL of ice-cold 70% ethanol. The cells can be stored in ethanol at -20°C for several weeks.
3. Centrifuge at 200g for 3 min, remove the ethanol, resuspend the cells in 5 mL of PBS, and centrifuge at 300g for 5 min.
4. Resuspend the pellet in 50 μL of a solution containing:
 - 10 μL TdT 5X reaction buffer.
 - 2.0 μL BrdUTP stock solution.
 - 0.5 μL (12.5 U) TdT.
 - 5 μL CoCl_2 solution.
 - 33.5 μL distilled H_2O .
5. Incubate the cells in this solution for 40 min at 37°C (see Notes 4 and 5).
6. Add 1.5 mL of the rinsing buffer, and centrifuge at 300 g for 5 min.
7. Resuspend the cell pellet in 100 μL of FITC-conjugated anti-BrdUrd MAb solution.
8. Incubate at room temperature for 1 h.
9. Add 1 mL of PI staining solution.
10. Incubate for 30 min at room temperature, or 20 min at 37°C , in the dark.
11. Analyze the cells by flow cytometry.
 - a. Illuminate with blue light (488-nm laser line or BG12 excitation filter).

- b. Measure green fluorescence of FITC at 530 ± 20 nm.
- c. Measure red fluorescence of PI at >600 nm.

Figure 1 (left-most panel) shows apoptotic cells detected by this method (see **Notes 6** and **7**).

3.1.2. DNA Strand Break Labeling with Other Markers for Analysis by Flow Cytometry

As mentioned in the **Subheading 1.**, DNA strand breaks can be labeled with deoxynucleotides tagged with a variety of other fluorochromes. For example, the Molecular Probes catalog lists seven types of dUTP conjugates, including three BODIPY dyes (e.g., BODIPY-FL-X-dUTP), fluorescein, cascade blue, Texas red and dinitrophenol. Several cyanine dyes conjugates (e.g., CY-3-dCTP) are available from Biological Detection Systems (Pittsburgh, PA). Indirect labeling via biotinylated or digoxigenin-conjugated deoxynucleotides offers a multiplicity of commercially available fluorochromes (fluorochrome-conjugated avidin or streptavidin, as well as digoxigenin antibodies) with different excitation and emission characteristics. DNA strand breaks, thus, can be labeled with a dye of any desired fluorescence color and excitation wavelength (**Fig. 1**).

The procedure described in **Subheading 3.1.1.** can be adapted to utilize each of these fluorochromes. In the case of direct labeling, the fluorochrome-conjugated deoxynucleotide is included in the reaction solution (0.25–0.5 nmol/50 μ L) instead of BrdUTP, as described in **step 4** of **Subheading 3.1.1.** Following the incubation step (**step 5**), omit steps 6–8 and stain the cells directly with PI (**step 9**). In the case of indirect labeling, digoxigenin- or biotin-conjugated deoxynucleotides are included in the reaction buffer (0.25–0.5 nmol/50 μ L) instead of BrdUTP at **step 4**. The cells are then incubated either with fluorochrome-conjugated antidigoxigenin MAb (0.2–0.5 μ g/100 μ L of PBS containing 0.1% Triton X-100 and 1% BSA) or with fluorochrome-conjugated avidin or streptavidin (0.2–0.5 μ g/100 μ L, as above) at **step 7** and then processed through **steps 8–10** as described in the protocol. Analysis is performed with excitation and emission wavelengths appropriate to the fluorochrome.

3.1.3. DNA Strand Break Labeling for Analysis by Laser Scanning Cytometry

1. Transfer 300 μ L of cell suspension (in tissue-culture medium with serum) containing approx 20,000 cells to a cytospin chamber. Cytoцентрифуге at 1000 rpm for 5 min.
2. Without allowing the cytospins to dry completely, prefix them in 1% formaldehyde in PBS for 15 min on ice.
3. Transfer the slides to 70% ethanol, and fix for at least 1 h. The cells can be stored in ethanol for weeks at -20°C .

4. Follow **steps 4–8 of Subheading 3.1.1**. Carefully layer small volumes (approx 100 μL) of the respective buffers, rinses, or staining solutions on the cytospin area of the horizontally placed slides. At appropriate times, remove these solutions with a Pasteur pipet (or vacuum suction pipet). To prevent drying, place $2 \times 4 \text{ cm}^2$ pieces of thin polyethylene foil on the slides over the cytospins, atop the drops of the solutions used for cell incubations (*see Note 8*).
5. Mount the cells under a coverslip in a drop of the PI staining solution. Seal the coverslip with melted paraffin or a gelatin-based sealer.
6. Measure cell fluorescence by laser scanning cytometry.
 - a. Excite fluorescence with a 488-nm laser line.
 - b. Measure green fluorescence of FITC at $530 \pm 20 \text{ nm}$.
 - c. Measure red fluorescence of PI at $>600 \text{ nm}$.Apoptotic cells detected by this method are shown in **Fig. 2**.

3.1.4. Controls

The procedure of DNA strand break labeling is rather complex and involves many reagents. Negative results, therefore, may not necessarily mean the absence of DNA strand breaks, but may be owing to methodological problems, such as loss of TdT activity, degradation of BrdUTP, and so forth. It is necessary, therefore, to include both positive and negative controls. An excellent control is to use HL-60 cells treated (during their exponential growth) for 3–4 h with 0.2 μM of the DNA topoisomerase I inhibitor camptothecin (CPT). Because CPT induces apoptosis selectively during S phase, cells in G_1 and G_2/M may serve as negative control populations, whereas the S phase cells in the same sample represent the positive control. Another negative control consists of cells processed as described in **Subheading 3.1.1.**, except that TdT is excluded from **step 4**.

3.2. Detection of Cells Incorporating BrdU by the SBIP Method

The method of DNA strand break labeling described above for the identification of apoptotic cells also can be used to detect the presence of BrdU or IdU incorporated into DNA. A variety of different schemes may be used to label cells with these precursors. Pulse labeling, for example, is used to detect S phase cells. A pulse-chase labeling strategy is used to follow a cohort of labeled cells progressing through various phases of the cycle for kinetic studies. Continuous labeling allows one to detect all proliferating cells in a culture or tumor to estimate the cell growth fraction. The scope of this chapter does not allow us to present technical details of cell labeling in cultures or in vivo, which are available elsewhere (17). In general, 10–30 μM BrdU are used for in vitro cell labeling, and the time of incubation for pulse-labeling varies between 10 and 60 min. It is important to maintain light-proof conditions (e.g., the cultures should be wrapped in aluminum foil) during and after cell labeling with BrdU, to prevent DNA photolysis.

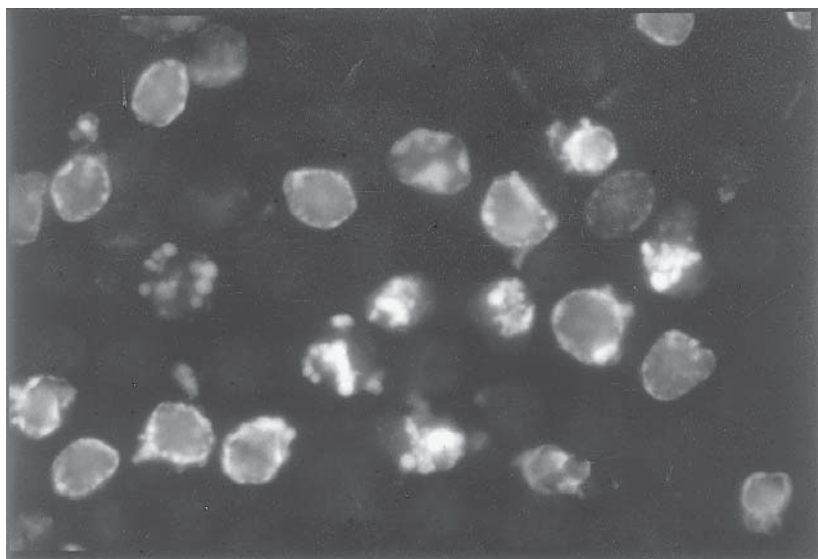


Fig. 2. Detection of apoptosis-associated DNA strand breaks. HL-60 cells were incubated with $0.15 \mu\text{M}$ camptothecin for 2.5 h, cytocentrifuged, fixed, and the DNA strand breaks were labeled with BrdUTP. The incorporated BrdU was then detected by FITC-conjugated anti-BrdU MAb, as described in **Subheading 3.1.3.**, however, the cells were not counterstained with PI. Note the predominance of DNA strand breaks in early apoptotic cells (prior to nuclear fragmentation) at the nuclear periphery, and strong labeling of the fragmented nuclei of late apoptotic cells.

Following incorporation of BrdU (or IdU), the cells are fixed, subjected to UV light illumination to photolyze the DNA at sites of the incorporated precursor, and the resulting DNA strand breaks are labeled identically to the DNA strand breaks of apoptotic cells in **Subheadings 3.1.1.** and **3.1.2.** To distinguish between DNA strand breaks in apoptotic cells and photolytically generated (BrdU-associated) breaks, the apoptotic DNA strand breaks may initially be labeled with a fluorochrome of one color, the cells then subjected to UV light illumination, and the photolytically generated breaks subsequently labeled with a fluorochrome of another color (**15**) (see **Subheading 3.1.2.**). The method of DNA photolysis presented below can be applied to any type of cells that have been labeled with BrdU or IdU.

3.2.1. SBIP Procedure for Cell Analysis by Flow Cytometry

1. Suspend $1\text{--}2 \times 10^6$ cells, previously incubated with BrdU, in 2 mL of ice-cold PBS.
2. Transfer the cell suspension to 60×15 mm polystyrene Petri dishes.

3. Place the dishes directly on the glass surface of a Fotodyne UV 300 analytic DNA gel transilluminator, which provides maximal illumination at 300-nm wavelength. Check the intensity of UV light by using a UV light photometer placed on the surface of the transilluminator. With relatively new UV bulbs, the intensity is expected to be 4–5 mW/cm². Other sources of UV light may be used provided that maximal intensity is at a wavelength close to 300 nm and the geometry of cell illumination favors uniform exposure of all cells (*see Note 9*).
4. Expose the cells to UV light for 5–10 min.
5. Transfer the cells to polypropylene tubes, and centrifuge at 300g for 5 min.
6. Suspend the cell pellet in 0.5 mL of PBS.
7. Transfer the cell suspension with a Pasteur pipet to a 6-mL polypropylene tube containing 4.5 mL of 70% ethanol, on ice. The cells can be stored in ethanol at –20°C for months.
8. Label the strand breaks, and process the cells for flow cytometry as described in **Subheading 3.1.1., steps 3–11**.

Controls should include cells incubated in the absence of BrdU (or IdU), as well as cells not illuminated with UV light.

3.2.2. SBIP Procedure for Cell Analysis by Laser Scanning Cytometry

1. Transfer 300 μ L of cell suspension in tissue culture medium (with serum) containing approx 20,000 cells into a cytospin chamber. Cytoцентрифуге at 1000 rpm for 6 min.
2. Without allowing the cytopspins to dry completely, fix the slides in 70% ethanol, in Coplin jars, on ice, for at least 2 h. The slides can be stored in ethanol for months at –20°C.
3. Rinse the slides in PBS.
4. To photolyze the DNA, remove the slides from PBS and place (while still wet) on the glass surface of the transilluminator. The cytopspinned cells should be placed face down, with the slide supported on both sides (e.g., with two other microscope slides) to prevent contact between the cells and the transilluminator glass surface (*see Note 10*).
5. Expose the cells to UV light for 5–10 min.
6. Process the cells as described in **steps 4 and 5 of Subheading 3.1.3**.
7. Measure cell fluorescence by laser scanning cytometry as described in **step 6 of Subheading 3.1.3**. (*See Figs. 3 and 4.*)

3.2.3. Controls for SBIP

As a negative control, analyze cells that were not incubated with BrdU (or IdU). Such a control is preferred over using an isotypic IgG (as a control for anti-BrdU MAbs), since the latter does not always allow accurate discrimination between BrdU-labeled and unlabeled cells. Two types of positive controls are suggested. As a positive control for the DNA strand break labeling procedure alone, apoptotic cells prepared using CPT as described in **Subheading 3.1.4**.

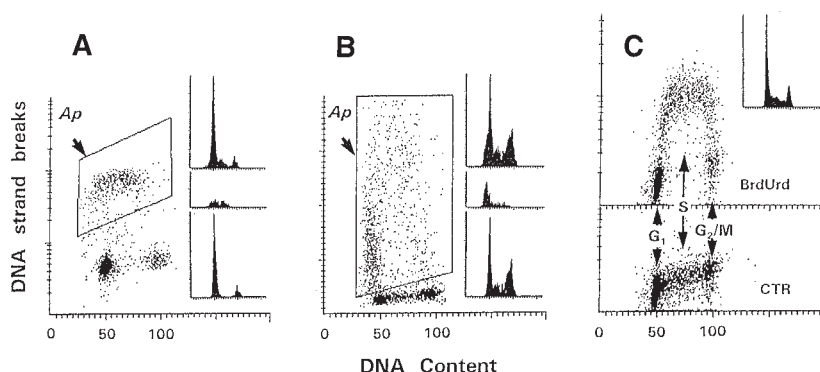


Fig. 3. Detection of apoptosis and DNA replication by differential labeling of DNA strand breaks and fluorescence measurement by laser scanning cytometry. Bivariate distributions (scattergrams) representing intensity of DNA strand break labeling with different fluorochromes vs cellular DNA content, identifying apoptotic, and BrdU-incorporating cells. (A) HL-60 cells were incubated with $0.15 \mu\text{M}$ camptothecin for 3 h. DNA strand breaks were directly labeled with dUTP conjugated to BODIPY. DNA histograms (insets) represent all cells (top), apoptotic cells located within the gating window (middle), and nonapoptotic cells (bottom). Notice that apoptosis is specific to S phase cells. Ordinate, exponential scale. (B) Cells were subjected to hyperthermia (43.5°C , 30 min) and then incubated for 3 h at 37°C . DNA strand breaks in apoptotic cells were indirectly labeled with d-dUTP and detected by fluoresceinated antidigoxigenin antibody. Top DNA histogram, all cells; middle histogram, apoptotic cells (within the gating window); bottom histogram, nonapoptotic cells. Ordinate, linear scale. (C) Detection of BrdU incorporation (1-h pulse) by SBIP using indirect labeling with d-dUTP and detection by fluoresceinated antidigoxigenin antibody (top). Bottom, the cells were incubated in the absence of BrdU (control). DNA histogram represents all cells. Ordinate, exponential scale (15).

should be used. As another positive control, exponentially growing cells incubated with $30 \mu\text{M}$ BrdU for 1 h, and then processed as described in **Subheading 3.2.1.** or **3.2.2.** should be used. In this control, one expects S phase cells, i.e., cells with a DNA content between 1.0 and 2.0 DNA index (DI), to show BrdU incorporation, and G_1 (DI = 1.0) and G_2/M cells (DI = 2.0) to be negative.

4. Notes

1. This method is useful for clinical material, such as obtained from leukemias, lymphomas, and solid tumors (18,19), and can be combined with surface immunophenotyping. The cells are first immunophenotyped, then fixed with formaldehyde (which stabilizes the antibody bound on the cell surface), and sub-

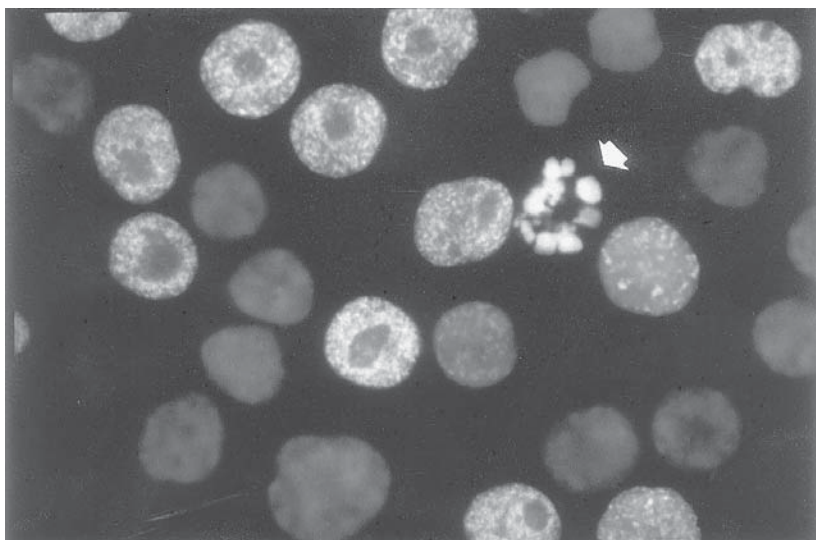


Fig. 4. Detection of photolysis-associated DNA strand breaks. HL-60 cells were pulse-labeled (1 h) with BrdU. Their DNA was photolyzed by exposure to UV light, and DNA strand breaks were labeled with BrdUTP as described in **Subheading 3.2.2.** Cellular DNA was counterstained with 7-aminoactinomycin D. The sites of DNA replication (“replication factories”) have a characteristic distribution, and nucleoli are unlabeled. A single apoptotic cell with a fragmented nucleus (arrow) is also labeled, but the labeling is diffuse, not granular.

- sequently subjected to the DNA strand break detection assay using different color fluorochromes (*see Subheading 3.1.2.*) than those used for immunophenotyping.
2. When the sample initially contains a small number of cells, cell loss during repeated centrifugations is a problem. To minimize cell loss, polypropylene or siliconized glass tubes are recommended. Since transferring cells from one tube to another results in irreversible electrostatic attachment of a large fraction of cells to the surface of each new tube, all steps of the procedure (including fixation) should be done in the same tube. Addition of 1% BSA to rinsing solutions also decreases cell loss. When the sample contains very few cells, carrier cells (e.g., chick erythrocytes) may be included, which later can be recognized based on differences in DNA content. Cell analysis by LSC, of course, has no such problem.
 3. Cell prefixation with a crosslinking agent, such as formaldehyde, is required to prevent extraction of the fragmented DNA from apoptotic cells. This ensures that despite repeated cell washings, the DNA content of apoptotic cells (and with it, the number of DNA strand breaks) is not markedly diminished. No prefixation with formaldehyde is required to detect DNA strand breaks induced by photolysis (**Subheading 3.2.**).

4. Alternatively, incubate at 22–24°C overnight.
5. Control cells may be incubated in the same solution, but without TdT.
6. It is generally easy to identify apoptotic cells owing to their intense labeling with FITC conjugated anti-BrdU MAb. Their high fluorescence intensity often requires the use of the exponential scale (logarithmic amplifiers of the flow cytometer) for data acquisition and display (**Fig. 1**). As is evident in **Fig. 1**, because cellular DNA content of both apoptotic and nonapoptotic cell populations is measured, the cell-cycle distribution and/or DNA ploidy of these populations can be estimated.
7. While strong fluorescence, which indicates the presence of extensive DNA breakage, is a characteristic feature of apoptosis, weak fluorescence does not necessarily mean the lack of apoptosis. In some cell systems, DNA cleavage generates DNA fragments 50–300 kb in size and does not progress into internucleosomal (spacer) sections (**20**).
8. It is essential that the incubations are carried out in moist atmosphere to prevent drying at any step of the reaction. Even minor drying produces severe artifacts.
9. In the SBIP procedure, to detect incorporated BrdU or IdU, the critical step is to expose the cells to an optimal and uniform dose of UV light. During the exposure, therefore, the layer of cell suspension should be thin and the Petri dishes should be exposed while in a horizontal position. Local cell crowding at the edges of the dish should be avoided, since it introduces undesired heterogeneity during illumination. Because the intensity of UV light at the surface of the transilluminator is uneven, depending very much on the position of the UV bulb underneath the glass, the “sweet spot” of relatively uniform intensity has to be found with a UV photometer. The cells should then be placed at this position for irradiation. Overexposure induces photolysis of native DNA, which has no incorporated BrdU. The signal-to-noise ratio in the detection of BrdU is then decreased owing to a high fluorescence background of the BrdU-unlabeled cells. Illumination of cells in the presence of Hoechst 33258, a dye that via a resonance energy transfer mechanism additionally photosensitizes BrdU, increases labeling of the DNA that contains incorporated BrdU (**21**).
10. Alternatively, the cells may be photolyzed in suspension, prior to fixation, as described in the procedure for flow cytometry (**steps 1–4 of Subheading 3.2.1.**), then cytocentrifuged, fixed in ethanol, and processed for DNA strand break labeling.

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Immunoassay for Single-Stranded DNA in Apoptotic Cells

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1. Introduction

Specific and sensitive cellular markers are necessary for the detection and quantitative analysis of apoptosis. Identification of apoptotic cells by specific markers in histological sections is especially important for heterogeneous cell populations, such as occurs in normal and neoplastic tissues. Histochemical analysis of apoptosis in tissue sections is critical because morphological evaluation does not provide accurate counts of apoptotic cells, and biochemical analysis of DNA breaks gives no information about cell types undergoing apoptotic death.

In this chapter, a novel immunochemical method for the detection of apoptotic cells is described (**1–3**). Most investigators at the present time rely on terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining to detect apoptotic cells and to evaluate the role of apoptosis in disease (e.g., see Chapter 51). However, several studies demonstrated that TUNEL is not specific for apoptosis, because it also detects necrotic and autolytic types of cell death (**2,4**). The sensitivity of TUNEL is compromised, because it detects only late stages of apoptosis associated with the low-mol-wt DNA fragmentation (**2**). The application of TUNEL is also limited by the fact that in various cell types, apoptosis is not accompanied by internucleosomal DNA fragmentation and therefore is not detected by TUNEL. Therefore, a specific and sensitive cellular marker based on a different mechanism than TUNEL is needed to determine the role of apoptotic death in biology and pathology.

The method for the identification of apoptotic cells described here is based on the staining of cell suspensions and tissue sections with monoclonal antibodies (MAbs) to single-stranded DNA (ssDNA). The procedure includes three

steps: fixation, heating, and staining with MABs. The critical step is the heating of cells or sections, which is performed in conditions inducing DNA denaturation only in apoptotic cells. The selective thermal denaturation reflects decreased stability of DNA induced by the digestion of nuclear proteins during apoptosis. The fact that proteolysis is responsible for DNA denaturation was demonstrated by the elimination of staining in apoptotic cells reconstituted with histones and by the induction of staining in nonapoptotic cells treated with proteolytic enzymes (3). The effect of proteolysis on MAB staining is in agreement with the ability of histones to stabilize DNA against thermal denaturation (5) and with the digestion of histones during apoptosis (6).

The higher sensitivity of MAB staining compared to TUNEL reflects the different mechanisms of the two techniques. TUNEL detects low-mol-wt DNA fragmentation associated with late apoptosis, whereas MABs to ssDNA detect the early stages of apoptosis and stain apoptotic cells in the absence of low-mol-wt DNA fragmentation (2,3). These advantages of the MAB method are based on the fact that protease activation is an early and universal event in apoptosis (6). Importantly, in contrast with the TUNEL method, MABs to ssDNA are specific for apoptotic cell death and do not detect necrotic cells (1,2).

Initially, MABs to ssDNA were applied in our studies for the detection of DNA breaks induced by cytotoxic agents (7,8). Heating of fixed cells suspended in phosphate-buffered saline containing a low concentration of Mg^{2+} induced DNA denaturation in cells treated with alkylating agents, but did not affect DNA conformation in untreated cells. There was a linear relation between MAB binding and the loss of cell viability (9). The method proved to be useful for the analysis of DNA damage and repair in individual cells, for the detection of drug-resistant cell subsets, and made possible the discovery of intercellular transfer of drug resistance (10).

The critical role of Mg^{2+} ions for the detection of DNA damage with anti-ssDNA MABs was established in these studies (7,8). Heating of cells suspended in medium without Mg^{2+} induced DNA denaturation and antibody binding in both treated and untreated cells. In the presence of 0.5–1.25 mM $MgCl_2$, only less stable DNA with drug-induced breaks was denatured. Higher concentrations of Mg^{2+} decreased MAB binding in drug-treated cells. The effects of Mg^{2+} on DNA denaturation in fixed cells is consistent with the stabilization of DNA in solution against thermal denaturation, which is achieved by the neutralization of negative charges in phosphate groups (11).

Cell lines in which drug treatment did not induce apoptosis were used for the analysis of DNA breaks with MABs to ssDNA (9,10). Only after the technique was applied to chronic lymphocytic leukemia (CLL) cells was the MAB staining of apoptotic cells discovered (12). A subset of cells having a decreased

DNA content and exhibiting intense MAb fluorescence and typical apoptotic morphology was observed in cultures of CLL cells. Although MAb binding was significantly higher in apoptotic cells than in nonapoptotic cells with DNA breaks, it was important to develop conditions under which only DNA in apoptotic cells was denatured and stained with MABs to ssDNA. The selective denaturation of DNA in apoptotic cells was achieved by increasing the MgCl_2 concentration during heating to 2.5–5 mM (1,2).

It is important to note that MABs F7-26 and AP-13 are specific for DNA in single-stranded conformation, and that the conditions of DNA denaturation determine the type of cellular damage detected by the procedure (1–3,7–10). In environments that destabilize DNA (e.g., low ionic strength, acid treatment), normal DNA will be stained by these MABs. Heating performed under conditions that moderately stabilize DNA (e.g., low Mg^{2+} concentration) will induce staining of DNA with breaks, while under conditions inducing maximal DNA stability (e.g., high concentration of Mg^{2+}), only DNA in apoptotic cells will denature and bind the antibody. MAB binding is not associated with DNA replication as demonstrated by the absence of staining of S phase cells (1–3). Probably, the digestion of DNA-bound proteins, such as histones, in apoptotic cells induces a high level of DNA instability to thermal denaturation, which is not prevented with the neutralization of phosphate groups by Mg^{2+} . F7-26 and AP-13 differ with respect to antigenic determinant (deoxycytidine and thymidine, respectively) and the size of DNA in single-stranded conformation necessary for binding. F7-26 binds to smaller stretches of ssDNA, which may explain the shorter heating time and the lower temperature required to effect its binding to DNA in apoptotic cells.

The relation between the intensity of drug-induced cellular damage and the binding of the antibody to DNA is different in nonapoptotic and apoptotic cells. Antibody binding characterized by mean fluorescence intensity in the total cell population is proportional to the drug dose when DNA breaks are measured in nonapoptotic cells (9). In contrast, fluorescence of the antibody is similar in apoptotic cells at various drug doses, and only the number of apoptotic cells is varied as a function of drug dose (13,14). These observations are consistent with the notion that apoptosis is an all-or-none phenomenon, which once triggered induces a similar type of damage.

In conclusion, the immunoassay for ssDNA in apoptotic cells is a procedure based on the selective thermal denaturation of apoptotic DNA and the staining of cells with MABs highly specific for DNA in single-stranded conformation. The low stability of apoptotic DNA to thermal denaturation is induced by the digestion of DNA-bound proteins during early stages of apoptosis and, in contrast to DNA instability induced by breaks, is not prevented by the presence of Mg^{2+} in the heating medium. MABs to ssDNA provide a cellular marker

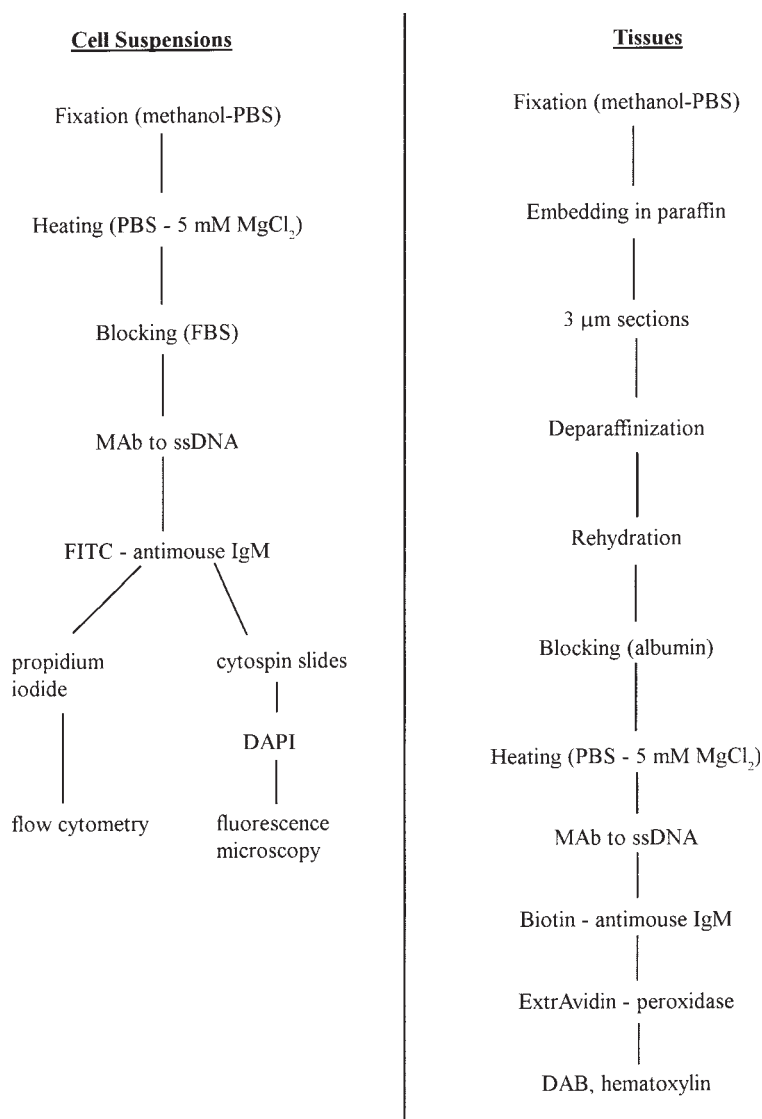


Fig. 1. Schematic diagram of the protocol for the staining of apoptotic cells with MAbs to ssDNA.

specific for apoptotic death that is independent of internucleosomal DNA fragmentation and useful for the detection of different stages of apoptosis in various cell types. The high sensitivity of the assay reflects the central role of proteolysis in the initiation and execution of apoptosis. The procedure is outlined in **Fig. 1**.

2. Materials

2.1. Staining of Cell Suspensions for Flow Cytometry and Fluorescence Microscopy

1. Phosphate-buffered saline (PBS): Dubecco's PBS without CaCl_2 and without MgCl_2 (Gibco BRL): 0.2 g KCl, 0.2 g KH_2PO_4 , 2.16 g Na_2HPO_4 , 8 g NaCl, distilled H_2O to 1 L, pH 7.2.
2. Methanol, 100%, precooled to -20°C .
3. 63 mM Magnesium chloride solution: Dissolve 6 mg of MgCl_2 (anhydrous, Sigma) per mL of dH_2O . Prepare fresh.
4. MAb F7-26 specific for ssDNA. Working concentration: 10 $\mu\text{g/mL}$ in PBS supplemented with 5% fetal bovine serum (FBS). Keep frozen at -20°C or -80°C . (APOSTAIN, Inc. [305]-868-3998; Fax: [305]-868-3445; E-mail: apostain@bellsouth.net; Website: www.apostain.com).
5. Fluorescein-conjugated goat antimouse IgM (Sigma): Working concentration: 1:50 in PBS supplemented with 5% FBS. Store frozen.
6. Propidium iodide, 1 $\mu\text{g/mL}$ in PBS. Store at 4°C in the dark. Stable for 4–8 wk.
7. 4'-6-Diamidino-2-phenylindole (DAPI), 0.1 $\mu\text{g/mL}$, in PBS. Store at 4°C in the dark.
8. Saccomano cytology collection fluid (Baxter).
9. Vectashield mounting medium for fluorescence (Vector).
10. S1 nuclease (Sigma). Working concentration: 100 U/mL in acetate buffer (0.03 M sodium acetate, 1 mM ZnSO_4 , pH 4.6). Store frozen at -20 or -80°C .
11. Histone solution: Type IIIS, lysine-rich fraction from calf thymus (Sigma), 0.25 mg/mL in PBS. Store frozen at -20°C .
12. Pyrex heavy-duty 15-mL centrifuge tubes or Kimble disposable 15-mL glass centrifuge tubes (Baxter).
13. Lauda circulating water bath M20 (Brinkman Instruments) or a hotplate.

2.2. Staining of Tissue Sections

1. Fixative: Methanol-PBS, 6:1. Store at -20°C .
2. Methanol, 100%.
3. Xylene.
4. Paraffin.
5. PBS (*see Subheading 2.1., item 1*).
6. 63 mM MgCl_2 (*see Subheading 2.1., item 3*).
7. 10% Triton X-100.
8. SafeClear tissue clearing agent (Curtis-Matheson).
9. Conical 50-mL polypropylene centrifuge tubes (Sarstedt).
10. 3% Hydrogen peroxide solution.
11. Bovine serum albumin (BSA): 0.1% in PBS.
12. MAb F7-26 specific to ssDNA (*see Subheading 2.1., item 4*).
13. Biotin-conjugated rat monoclonal antimouse IgM (Zymed): Working concentration: 1:50 in PBS supplemented with 0.2% Tween-20 and 0.1%, sodium azide. Store at 4°C . Stable for 2–3 mo. **Caution: Sodium azide is highly toxic.**

14. ExtrAvidin-peroxidase (Sigma): Stock solution 100 $\mu\text{g/mL}$ in PBS; store at -20°C . Working concentration: 10 $\mu\text{g/mL}$, prepare fresh.
15. Liquid DAB-plus substrate kit (Zymed).
16. Lerner Hematoxylin.
17. Mounting medium (Baxter).
18. Water bath (*see Subheading 2.1., item 13*).

3. Methods

3.1. Detecting ssDNA in Cell Suspensions

3.1.1. Fixation (*see Note 1*)

1. Centrifuge $1-2 \times 10^7$ cells at 200g for 5 min.
2. Decant, and resuspend the pellet in 1 mL of PBS.
3. Slowly add 6 mL of cold methanol while vortexing.
4. Store the fixed cells at -20°C for 16–24 h before staining.

3.1.2. Heating (*see Notes 2–5*)

1. Distribute $0.5-1.0 \times 10^6$ fixed cells into glass tubes, centrifuge, and decant the fixative.
2. Resuspend the pellet in 0.4 mL of PBS freshly supplemented with 5 mM MgCl_2 (9.2 mL of PBS + 0.8 mL of MgCl_2).
3. Immerse the rack with tubes into a circulating water bath (preheated to 99°C) for 5 min or into a beaker with boiling water on a hotplate for 5 min.
4. Place the rack in ice-cold water for 10 min.

3.1.3. Blocking (*see Note 6*)

Add 0.4 mL of 40% FBS in PBS and incubate on ice for 15 min.

3.1.4. Staining (*see Notes 6 and 7*)

1. Centrifuge the cells at 200g for 5 min.
2. Resuspend the pellet in 100 μL of MAb F7-26 solution.
3. Incubate at room temperature for 30 min.
4. Rinse twice in PBS.
5. Resuspend the pellet in 100 μL of FITC-conjugated antimouse IgM.
6. Incubate at room temperature for 30 min.
7. Rinse once in PBS.
8. For flow cytometry: Resuspend the pellet in 0.5 mL of propidium iodide solution.
9. For fluorescence microscopy: Resuspend the pellet in Saccomano fluid, stain cytospin slides with DAPI for 10 min, rinse with PBS, dry, and mount in VectaShield.

3.1.5. Analysis

1. Flow cytometry measurements are performed using log scale for green fluorescence from fluorescein-labeled antibody and linear scale for DNA-bound

propidium iodide. For example, FL 1 and FL 2 settings for FACScan were 440/log and 400/1.85, respectively, for etoposide-treated MOLT-4 cells. Typical fluorescence contour plots are presented in ref. (1,2).

2. Slides are observed in a fluorescence microscope using UV excitation for the DNA-fluorochrome DAPI and 450–490 nm excitation for fluorescein-labeled antibody. Dual-labeling makes it possible to characterize chromatin distribution in positive cells by changing excitation filters (1,2).

3.1.6. Controls (see **Notes 8–10**)

The following two controls are recommended:

1. Incubate the cells in histone solution for 30 min at room temperature before heating, that is, before **step 2** of **Subheading 3.1.2**. Reconstitution of apoptotic nuclei with lysine-rich histones completely suppresses MAb binding.
2. After heating, treat the cells with S1 nuclease at 37°C for 30 min. Digestion of ssDNA eliminates MAb binding. Buffer alone has no effect on the staining.

3.2. Detecting ssDNA in Tissue Sections

3.2.1. Fixation and Embedding (see **Notes 1, 11, and 12**)

1. Fix fresh tissue in methanol-PBS at –20°C for 1–3 d.
2. Dehydrate the fixed tissue in two changes of absolute methanol (1 h each) and two changes of xylene (1 h each).
3. Incubate in two changes of paraffin at 56°C (1 h each).
4. Embed in paraffin.
5. Cut 3- μ m sections from paraffin blocks.
6. Attach sections to superfrost/plus slides, and heat at 56°C for 1–2 h.

3.2.2. Deparaffinization and Rehydration (see **Notes 13 and 14**)

1. Incubate the slides in two changes of SafeClear (15 min each).
2. Incubate the slides in three changes of methanol-PBS (20 min each).
3. Rinse with PBS.
4. Incubate the slides in PBS supplemented with 0.2% Triton X-100 and 5 mM MgCl₂ for 5 min.

3.2.3. Heating (see **Notes 2–5**)

1. Transfer the slides into 50-mL centrifuge tubes containing 30 mL of room temperature PBS freshly supplemented with 5 mM MgCl₂ (27.6 mL PBS + 2.4 mL MgCl₂).
2. Immerse the rack containing the centrifuge tubes into a circulating water bath (preheated to 99°C) for 5 min or into a beaker of boiling water on a hotplate for 5 min.
3. Remove the slides with forceps from the centrifuge tubes, and transfer to tubes containing ice- cold PBS for 10 min.

3.2.4. Staining (see **Notes 6 and 7**)

1. Incubate the slides in 3% H₂O₂ for 5 min to block endogenous peroxidase activity.
2. Rinse twice with PBS.
3. Treat the slides with 0.1% BSA at room temperature for 30 min.
4. Rinse twice with PBS.
5. Apply anti-ssDNA MAb F7-26 to the top of the tissue section (100 µL/slide).
6. Incubate at room temperature for 15 min. Rinse twice with PBS.
7. Apply biotin-conjugated rat antimouse IgM for 15 min, and then rinse twice with PBS.
8. Apply ExtrAvidin-peroxidase for 15 min, and then rinse with PBS.
9. Apply chromogen solution (DAB), counterstain with hematoxylin, dehydrate, and mount.

3.2.5. Controls (see **Notes 8–10**)

The following four controls are recommended:

1. Following deparafinization (i.e., **steps 1–3 of Subheading 3.2.2.**), treat the tissue sections with proteinase K (2 µg/mL in PBS) at 37°C for 20 min, rinse with PBS and proceed to **step 4 of Subheading 3.2.2.** All nuclei are stained (positive control).
2. Heat the slides immersed in dH₂O rather than in PBS/MgCl₂ solution. All nuclei are brightly stained (positive control).
3. Following deparafinization, incubate the sections in histone solution for 20 min, rinse with PBS, and proceed to **step 4 of Subheading 3.2.2.** Staining of apoptotic nuclei is eliminated (negative control).
4. Rinse the heated sections with saline, treat with S 1 nuclease at 37°C for 20 min, rinse with PBS, and proceed to **step 1 of Subheading 3.2.4.** Staining of apoptotic nuclei is eliminated (negative control).

4. Notes

1. Fixation in methanol-PBS produces optimal results. The fixative should be cooled to –20°C before addition to cells and tissues. Fixed material should be kept in freezer. Fixation of tissues at room temperature or in refrigerator must be avoided.
2. PBS supplemented with MgCl₂ should be prepared shortly before heating by mixing the stock solution of MgCl₂ with PBS. PBS supplied by Gibco BRL is recommended at least at the initial stage of application. MgCl₂ should be kept anhydrous, because the concentration of Mg²⁺ is critical for the specific staining.
3. Clean glass centrifuge tubes should be used for the heating of cell suspensions. The types of tubes should not be changed because the thickness of glass affects the process of heating. For the heating of slides with tissue sections, disposable polypropylene centrifuge tubes can be used. The temperature of the PBS/MgCl₂ solution inside the tube with slides after 5 min of heating was found to be 8–9°C lower than the temperature in the water bath. The heating regimens described here were selected for MAb F7-26 and for the specific conditions (type of tubes,

- volume of fluid) indicated in the protocol. Although the exact time and temperature may have to be determined for the particular experimental conditions, the heating should be in the range of 99–100°C for 5 min.
4. A heating, circulating water bath with electronic temperature control and digital display is recommended, especially when large numbers of tubes are heated. Heating also may be performed by immersion of a rack with a small number of tubes into a beaker or vessel containing boiling water on a hotplate, or in a noncirculating water bath with boiling water. Tubes with cells or slides should always be kept in a rack during heating. In general, specific staining will be absent after insufficient heating, whereas excessive heating will induce DNA denaturation in nonapoptotic cells and produce nonspecific background staining.
 5. For the optimal staining of cell suspensions, carefully remove the fixative, add PBS/MgCl₂, and heat the tubes as soon as possible. Rinsing of fixed cells in PBS before heating and delay between the addition of PBS/MgCl₂ and heating may decrease specific staining. The volume of fluid in which the cells are suspended during heating and the number of cells should be kept constant for reproducible results.
 6. Blocking and an optimal concentration of the MAb to ssDNA are needed to obtain specific staining of apoptotic cells. Nonspecific binding of MAb F7-26 to methanol-fixed cells is blocked by FBS, whereas BSA is the best blocking agent for tissue. Bright staining of apoptotic cells and the absence of antibody binding to nonapoptotic cells is obtained with the recommended range of MAb F7-26 concentrations (1–3). Excessive concentration of the antibody may induce some nonspecific binding to nonapoptotic cells, although at all concentrations, the staining of apoptotic cells will be more intense.
 7. Second-step reagents should not bind to methanol-fixed cells or tissues not treated with MAbs to ssDNA. Fluorescein-labeled antimouse IgM in PBS containing FBS or newborn calf serum is recommended for the staining of cell suspensions. The concentration and the type of serum that are needed to suppress the nonspecific binding of the antimouse antibody to methanol-fixed cell suspensions may vary, depending on the cell and antibody type. Biotin-labeled rat monoclonal antimouse IgM and ExtrAvidin-peroxidase are optimal second-step reagents for paraffin sections of methanol-fixed tissues.
 8. The following positive controls are recommended to determine the sensitivity of MAb staining. Treatment of cells and tissue sections with a proteolytic enzyme before heating in MgCl₂-supplemented PBS should induce staining of nonapoptotic cells. This procedure reproduces DNA instability induced by the digestion of nuclear proteins during apoptosis. Heating of cells or sections suspended in dH₂O should induce bright staining of all nonapoptotic nuclei, indicating that the procedure is adequate for the detection of denatured DNA. Cell suspensions from nontreated cultures or sections of tissues with low apoptotic indices are used for the positive controls. Cells in crypts of small intestine that are negative after standard staining should be positive in proteinase- treated or dH₂O-heated sections.

9. Two procedures are recommended to determine that the staining of apoptotic cells reflects the exposure of single-stranded regions in DNA destabilized by the digestion of DNA-bound proteins. Elimination of staining by S 1 nuclease demonstrates that only ssDNA is detected by MAb binding. Reconstitution of cells or sections with lysine-rich histones restores DNA stability in apoptotic cells and prevents DNA denaturation during heating. Cells or sections with high apoptotic indices should be used for the S 1 nuclease and histone negative controls.
10. The following experimental models are recommended to evaluate the staining of apoptotic cells with MAbs to ssDNA:
 - a. Exponentially growing MOLT-4 cultures treated with 5 μ M etoposide for 6 h.
 - b. Monolayer cultures of MDA-MB-468 breast cancer cells treated with 0.5 μ M staurosporine for 2–4 h or 15 μ M cisplatin for 18 h. Floating cells with apoptotic morphology are stained by the MAbs, whereas 10–20% of attached cells at early stages of apoptosis are positive.
 - c. Small intestine from untreated or hydroxyurea-treated mice (500 mg/kg 4 h). Surface villous cells are positive in control tissue, but crypt cells with condensed and fragmented chromatin are stained in drug-treated mice.
 - d. Mouse thymus removed 5 h after the injection of 100 mg/kg methylprednisolone.
11. The thickness of tissue specimens should not be more than 3–5 mm, because sections from poorly fixed tissues will not be stained. Incubation in xylene may be longer for larger specimens and should be continued until the tissue becomes clear.
12. Freshly cut sections should be used for best results. Background nonspecific staining may develop in sections after prolonged storage.
13. Tissue sections and cells should be kept moist at all steps of the procedure. Air-dried sections, cryostat sections, smears, and cytopsin preparations are not suitable for staining with anti-ssDNA MAbs, because drying will prevent selective denaturation of DNA in apoptotic cells.
14. The use of SafeClear as a substitute for xylene for the deparaffinization of tissue sections is recommended. SafeClear is nontoxic and produces better results than xylene. Prolonged treatment of sections in methanol/PBS is needed to remove SafeClear before rehydration in PBS.

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