METHODS IN MOLECULAR BIOLOGY™ 313

Yeast Protocols

Second Edition

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HUMANA PRESS

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HUMANA PRESS TOTOWA, NEW JERSEY

999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

www.humanapress.com

This publication is printed on acid-free paper. ♾
ANSI Z39.48-1984 (American Standards Institute) Permanence of Paper for Prined Library Materials.

Production Editors: Nicole E. Furia and Melissa Caravella
Cover design by Patricia F. Cleary
Cover illustration: From Fig. 2 in Chapter 10, "Yeast Fluorescence Microscopy," by Jiří Hašek.

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Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

eISBN 1-59259-958-3

ISSN 1064-3745

Library of Congress Cataloging-in-Publication Data

Yeast protocols / edited by Wei Xiao. -- 2nd ed.
p. ; cm. -- (Methods in molecular biology ; 313)
Includes bibliographical references and index.
ISBN 1-58829-437-4 (alk. paper)
1. Yeast--Biotechnology--Laboratory manuals. 2. Yeast fungi --Laboratory manuals. I. Xiao, Wei, 1957 Apr. 10- . II. Series: Methods in molecular biology (Clifton, N.J.) ; 313.
[DNLM: 1. Yeasts--physiology--Laboratory Manuals. 2. Yeasts --cytology--Laboratory Manuals. 3. Yeasts--isolation & purification --Laboratory Manuals. W1 ME9616J v.313 2006 / QW 25 Y415 2006]
TP248.27.Y43Y416 2006
579.5'63--dc22

2005006244

Preface

Unicellular yeast cells have been traditionally used as models of lower eukaryotic organisms and the study of yeast has made tremendous contributions to our understanding of life and cellular metabolism. In particular, the budding yeast *Saccharomyces cerevisiae* is the first organism whose entire genome sequence was determined. This has greatly facilitated and expedited our efforts aiming at deciphering functions of the entire genome of approximately 6200 genes. As a consequence, the functionally unknown genes have decreased from two-thirds of the genome in 1994 to less than 40% today. We are confident that in another decade, the functions of the vast majority of yeast genes will be uncovered, with new functions added to previously described genes as well.

Technological advances are the major force driving yeast research in a race that out-competes perhaps any other rival organisms. Since publication of the first edition of *Yeast Protocols* in 1996, many new techniques have been invented and original protocols improved or refined. This second edition should serve as a stand-alone protocols handbook suitable for daily use in research laboratories. It includes recent advanced protocols in addition to the major basic techniques. Hence, both yeast research laboratories and those researchers who wish to use yeast as a host to study their favorite genes from other organisms will find this book useful.

Chapter 1 serves as a start-up kit for those who are not yet experienced with yeast to learn basic handling techniques. Chapters 2–6 describe how to isolate subcellular components, including organelles and macromolecules. Chapters 7–11 contain a collection of protocols for basic cellular and molecular analysis specific for yeast cells.

Perhaps the greatest advantages of using budding yeast for genetic analysis are its powerful genome manipulation and mutant selection systems. Chapters 12–15 describe both traditional and advanced protocols, as well as novel approaches that create conditional mutant phenotypes. Chapters 16–23 contain a series of protocols that were essentially invented in yeast cells to study genetic interactions, DNA and chromatin metabolism, and gene expression. I want to point out that some of the protocols in the above chapters are challenging, and may take time to develop proficiency in, but the authors have done an excellent job of providing sufficient details to make them reproducible. Protocols in the last four chapters aim to study foreign genes and gene products in yeast cells, although they can also be used to analyze native yeast genes and gene products.

Finally, I wish to take this opportunity to thank all authors for their initial commitment, cooperation, and contributions that made my first editing job a pleasant experience. I also wish to express my sincere thanks to Michelle

Hanna, known by other authors as “an internal reviewer,” for her outstanding editing, and to Shirley Cooke for her excellent editorial assistance. Dr. John Walker made great efforts in providing guidance and encouragement. Without their assistance, this book might not exist.

Wei Xiao

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1

Basic Investigations in *Saccharomyces cerevisiae*

Brendan P. G. Curran and Virginia Bugeja

Summary

This chapter aims to provide the reader with a one-stop reference to the basic procedures needed to grow, store, mate, and sporulate yeast cells. Starting with recipes for the different types of media, the chapter then goes on to explain how cells are grown to the appropriate cell numbers at the correct stage in the growth cycle. It also provides a detailed explanation on both short- and long-term storage of yeast cells. It then explains how to set up genetic crosses, before finally dealing in some detail with the demanding technique of diploid cell sporulation and spore isolation. It ends with an introduction to the Internet-based yeast resources, which are becoming increasingly important in the investigation of *Saccharomyces cerevisiae* in the post-genomic era.

Key Words: *Saccharomyces cerevisiae*; growth media; storage; genetic crosses; sporulation.

1. Introduction

A model organism and the first eukaryote to have its genome sequenced *Saccharomyces cerevisiae* had been at the forefront of eukaryotic cellular and molecular biology for more than 50 yr. With its basic genetics, biochemistry, and cellular biology established many decades ago, *S. cerevisiae*'s autonomously replicating plasmid, whole cell transformation system, and the ability rapidly to form discrete colonies on simple defined media ensured that it remained at the forefront of developments during the recombinant DNA revolution ***(1)***. However, after entering the history books in 1996 ***(2)***, yeast genetics and molecular biology came of age. The DNA sequence of *S. cerevisiae* was just the starting point for large-scale molecular analysis of this eukaryotic cell. Within a very few years, this extremely tractable model organism rapidly yielded up a whole series of molecular secrets on a global scale: each of its genes was systematically deleted in search of phenotypes ***(3)***, technology to

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

allow its global mRNA profiles to be identified was developed ***(4)***, and all possible protein–protein interactions were examined ***(5)***. Much more than this, however, *S. cerevisiae* became a central player in the development of an entirely new approach to biological research: systems biology ***(6)***. This newly emerging field uses a cross-disciplinary approach to develop working computer models of how molecules interact to generate biological phenomena. In short, this simple eukaryote is uniquely placed to address many questions of fundamental biological importance and has become a central player in post-genomic research.

This revolution in yeast bioinformatics means that basic yeast investigations have become as much a matter of accessing relevant World Wide Web addresses as how to manipulate yeast cells, and indeed much of the following information is currently available on the Internet ***(7)***. Nevertheless this chapter is offered for the benefit of researchers who would like access to the basic tricks of the trade as accumulated by two workers with more than 50 person-years between them working with this exciting eukaryotic cell.

1.1. S. cerevisiae*: Nomenclature*

One of the great attractions of this yeast as a model organism ***(7,8)*** is that it is extremely genetically tractable and can exist as either haploid or diploid cells. Haploid cells are of one of two mating types designated Mat**a** or Matα. Such cells can be grown by repeated subculturing for many generations and stored indefinitely under appropriate conditions. Haploids of opposite mating-type mate quite readily to produce diploid cells that are also stable and can be grown and stored as aforementioned. Diploid cells can be induced to undergo sporulation by growth in the absence of nitrogen, forming four-spored asci after 7–10 d. The products of a single meiotic event, asci contain two Mat**a** and two Matα haploid cells.

Whenever yeast strains are described in the literature, ploidy status and genetic markers are defined. For example, strain MTC47 (MAT**a** *leu2-3,112 ura3, his3-Δ1 trp1::LEU2*) is a haploid strain of mating type "**a**," carrying an allele of the *leu2* gene with two point mutations (3 and 11), a point mutation in the *URA3* gene, a deletion of the *HIS*3 gene and a wild-type *LEU2* gene inserted into *TRP1* gene causing it to become a trp1 mutant. Thus dominant alleles are denoted by using uppercase italics for all letters of the gene symbol, e.g., *URA3*, whereas lower-case letters denote the recessive allele (*ura3*). Wild-type genes are designated with a superscript "plus" (this strain is wild-type for all other genes, e.g., $ADE3^{+}$).

2. Materials

2.1. Preparing Growth Media

1. *See* **Tables 1–3**.
2. Flasks.
3. Sponge bungs and tin foil.
4. Agar.
5. Sterile Petri dishes.

2.2. Maintaining Stocks of Yeast Strains

1. Yeast strain to be maintained.
2. 2 YEPD plates.
3. 10 Sterile 20-mL universal containers.
4. 120 mL Sterilized molten YEPD containing 2% (w/v) agar.
5. 20 mL Sterile liquid YEPD containing 15% (w/v) glycerol.
6. A few small sterile (1.5–5.0-mL) cryotubes.

2.3. Growing Yeast Cells in Liquid Media

1. 10 mL of the appropriate medium (*see* **Table 1**) autoclaved in a 50-mL flask (*see* **Note 11**).
2. 100 mL of the same medium autoclaved in a 500-mL flask.
3. A pure culture of the yeast strain in question.
4. A spectrophotometer set at 600 nm.

2.4. Mating Yeast Cells

1. Small 10 mL overnight YEPD cultures of both strains (Strains A and B).
2. 20 mL of sterile water.
3. Sterile Eppendorf tubes and pipet tips.
4. A plate of the appropriate selective medium (i.e., A or B alone cannot grow but the diploid can) (*see* **Note 14**).

2.5. Sporulation and Spore Isolation

1. The diploid strain to be sporulated.
2. 15 mL of presporulation and sporulation media (**Table 4**) in 250-mL flasks.
3. 100 mL of sterile water.
4. β-glucuronidase.
5. 20 mL of autoclaved mineral oil.
6. Nonselective plates.
7. Appropriate selective plates.

Table 1
Basic Media

Medium	Ingredient	Per liter
YEPD (A complex rich medium)	Yeast extract	10 g
	Peptone	20 g
	Glucose	20 g
	Distilled water (to 1 L)	
Defined minimal medium		
Commercial source	Yeast nitrogen base (without amino acids)	6.7 g
	Glucose	20 g
	Amino acids/nucleotides (**Table 3**)	As required
	Distilled water (to 1 L)	
From first principles[a]	Potassium phosphate buffer	10 mL
	Calcium chloride	1 mL
	Other salts	20 mL
	Amino acids/nucleotides (**Table 3**)	As required
	Distilled water (to 1 L)	
	Autoclave and then add:	
	Vitamins I	1 mL
	Vitamins II	1 mL
	Trace elements I	1 mL
	Trace elements II	1 mL
	Ferric chloride	2 mL

[a]Adapted from **ref. *9***. Volumes in final column refer to volumes from the stock solutions in **Table 2**.

3. Methods

3.1. Preparing Growth Media

As a general rule, yeast cells grow most rapidly at 28–30°C in rich YEPD medium (*see* **Table 1**). Wild-type cells require appropriate sources of carbon (normally glucose) and nitrogen (normally ammonium sulphate), and a few basic minerals, vitamins, and salts. Defined minimal medium containing these can be bought from a number of commercial outlets as pre-prepared dehydrated media (*see* **Table 1**). It can also be made from first principles in the laboratory (*see* **Tables 1** and **2**) but normally this is not necessary. The most commonly used laboratory yeast strains carry one or more mutations in meta-

Table 2
Stock Solutions for Defined Minimal Medium

Stock solution	Volume prepared	Constituents	Weight	Preparation and storage
Potassium phosphate buffer	1 L	Potassium phosphate (monobasic)	85 g	Autoclave and store at room temperature.
		Potassium phosphate (dibasic)	15 g	
Calcium chloride	100 mL	Calcium chloride	10 g	Autoclave and store at room temperature.
Other salts	100 mL	Ammonium sulphate	25 g	Autoclave and store at room temperature.
		Sodium chloride	0.5 g	
		Magnesium sulphate	2.5 g	
Vitamins I	50 mL	Biotin	1 mg	Filter-sterilize and store in 1-mL aliquots at –20°C
		Calcium pantothenate	100 mg	
		Inositol	500 mg	
		Pyridoxine hydrochloride	20 mg	
		Thiamin hydrochloride	20 mg	
Vitamins II	50 mL	Folic acid	0.1 mg	Filter-sterilize and store in 1-mL aliquots at –20°C
		p-Aminobenzoic acid	10 mg	
		Niacin	20 mg	
		Riboflavin	10 mg	
Trace elements I	50 mL	Boric acid	25 mg	Filter-sterilize and stored in 1-mL aliquots at –20°C
		Copper sulphate	2 mg	
		Zinc sulphate	20 mg	
		Potassium iodide	5 mg	
Trace elements II	50 mL	Manganese sulphate	20 mg	Filter-sterilize and stored in 1-mL aliquots at –20°C
		Sodium molybdate	10 mg	
Ferric chloride	50 mL	Ferric chloride	10 mg	Filter-sterilize and store in 1-mL aliquots at –20°C.

bolic genes, e.g., Strain MTC mentioned previously requires Uracil, Histidine, and Tryptophan supplements if it is to grow in defined minimal medium. It is frequently necessary, therefore, to supplement defined minimal media with missing metabolic product(s) (*see* **Table 3**). This is referred to as supplemented minimal medium. Complete minimal medium is simply defined minimal medium containing all of the supplements in **Table 3**. Complete minimal media lacking one or more of these are referred to as drop-out media.

Table 3
Volumes of Stock Solutions Added to Supplement Defined Minimal Medium[a]

Constituent	Volume of stock per 1 L of medium	Weight of constituent in a 100 mL stock solution	Final concentration in the complete medium (mg/L)
L-Tryptophan	2 mL	1 g	20
L-Histidine-HCl	2 mL	1 g	20
L-Arginine-HCl	2 mL	1 g	20
L-Methionine-HCl[b]	2 mL	1 g	20
L-Leucine	3 mL	1 g	30
L-Isoleucine	3 mL	1 g	30
L-Lysine-HCl	3 mL	1 g	30
L-Phenylalanine	5 mL	1 g	50
L-Valine	5 mL	3 g	150
L-Serine	5 mL	8 g	400
L-Threonine[b]	5 mL	4 g	200
L-Glutamic Acid	10 mL	1 g	100
L-Aspartic Acid[b]	10 mL	1 g	100
Uracil	10 mL	200 mg	20
Adenine sulphate	10 mL	200 mg	20
L-Tyrosine	15 mL	200 mg	30

[a]Complete minimal medium contains all of these. 100 mL stock solutions of each component are prepared in distilled water, autoclaved, and stored at room temperature.

[b]Constituent should be filter-sterilized using a 45-µm filter, and added to the medium after it has been autoclaved.

1. For liquid media, mix the constituents with distilled water in a flask that holds twice the required volume of medium.
2. For solid plates, add 2% (w/v) agar to the liquid in the flask and shake to disperse prior to autoclaving.
3. Plug the flask with a foam bung or nonabsorbent cotton wool.
4. Cover the bung with tin foil to keep it dry.
5. Autoclave at 121°C at 15 psi (1 atmosphere) for 15 min.
6. Open the autoclave after it has cooled sufficiently to reach zero pressure.
7. Remove flasks using gloves and allow to cool.
8. Plates can be poured when the medium (*see* **Notes 1–4**) has reached approx 50°C. (The flask can be held in bare hands without discomfort.)
9. Gently swirl the agar-containing medium to ensure agar dispersal (avoid introducing bubbles) and then pour 20–25 mL into each sterile Petri dish.
10. Allow to set and then dry for 2 d at room temperature.
11. Store at 4°C in the plastic bags from which they came.

Table 4
Sporulation Media

Medium	Ingredient	Per liter
Presporulation medium	Yeast extract (0.8%)	8 g
	Bacto-peptone (0.3%)	3 g
	Glucose (10%)	100 g
	Distilled water (to 1 L)	
Sporulation	Potassium acetate (1%)	10 g
	Yeast extract (0.1%)	1 g
	Glucose (0.05%)	0.5 g
	Distilled water (to 1 L)	

3.2. Maintaining Stocks of Yeast Strains

1. Streak the cells out on one YEPD plate and incubate for 2–3 d at 28–30°C to obtain single colonies.
2. Use one colony to streak out a number of patches of cells on the second YEPD plate (*see* **Notes 5** and **6**).
3. Pour 10 mL of the sterilized molten YEPD into the sterile 20-mL containers under aseptic conditions and place them at an angle so that the medium is just below the neck of the container before allowing them to set.
4. Add an appropriate volume of the glycerol-containing YEPD to the cryotubes.
5. Thickly innoculate the slopes using the cells from the patches. Incubate at 28–30°C overnight.
6. Store at 4°C. Most strains last for 6–12 mo under these conditions (*see* **Notes 7** and **8**).
7. Transfer large numbers of cells using sterile applicator sticks/loops into the YEPD plus glycerol in the cryotubes.
8. Store below –60°C. Strains can be maintained indefinitely at this temperature (*see* **Notes 9** and **10**).

3.3. Growing Yeast Cells

Yeast cells are not difficult to grow, but their growth requirements can vary greatly depending on their genetic background and intended use. As a general rule, yeast cells are grown most easily at 28–30°C on rich complex media (YEPD) containing 1% w/v yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose (*see* **Table 1**). The growth of newly inoculated cells (at 2×10^5/mL) follows a typical growth curve: a lag phase of two to three cell divisions over a 5-h period, followed by exponential growth for six more divisions giving approx $4–6 \times 10^7$ cells/mL, before they undergo a shift to ethanol respiration over approx two more divisions as they enter stationary phase.

Wild-type cells can also be grown on minimal media. These can be prepared from first principles in the laboratory or bought in as pre-prepared dehydrated media (*see* **Table 1**). The most commonly used laboratory yeast strains carry one or more mutations in metabolic genes. Many also harbor plasmids that need to be selected for in order to maintain them. It is sometimes necessary, therefore, to alter the carbon source, or more frequently, supplement defined minimal media with the missing metabolic product (most commonly one or more amino-acids/nucleotides).

Most laboratory haploid strains have a doubling time of approx 1.5 h in complete YEPD medium and approx 2.5 h in complete minimal media during exponential growth at 28–30°C.

3.3.1. Growing Yeast Cells in Liquid Media

1. Using aseptic technique, inoculate the starter culture with a loopful of yeast cells.
2. Transfer this flask to a 28–30°C shaking water-bath overnight.
3. On the next day, blank the spectrophotometer using the appropriate sterile medium.
4. Using aseptic technique, remove a small volume of the starter culture into a cuvet and measure the absorbance of the cells at 600 nm (OD_{600}). Use dilutions to ensure that the spectrophotometer is in the linear range (<0.6 on our machines).
5. Calculate the number of cells/mL. OD_{600} of 0.1 is approx $1–2 \times 10^6$ cells/mL (*see* **Notes 12** and **13**).
6. Using aseptic technique, inoculate the main culture with the appropriate volume of the starter culture. This depends on the type of medium, the cell division time, and the number of cells required the next day. For example, an inoculum that provides 1×10^4 cells/mL in the large culture will grow to mid-exponential growth phase $2–4 \times 10^6$ (0.2 OD_{600}) the next morning, assuming a 2.5-h division time during 20 h of growth in complete minimal medium.

3.4. Mating Yeast Cells

The well-defined and extremely useful yeast mating system can be exploited for any one of a number of reasons. These include: combining genetic markers from different strains, testing the mating type of an unknown strain, or investigating whether a newly isolated mutant is allelic to an already existing strain. Matings require haploid strains of opposite mating type and they can be undertaken in liquid or on solid media. The mating process normally takes 4–6 h and the resulting zygotes can normally be identified microscopically at this time. Skilled practitioners can isolate such zygotes using a micro-manipulator; however, diploids can be identified as colonies growing on appropriate selective media when the haploid parent strains carry complementary genetic markers. A simple plate-based complementation method is described below ***(10)***.

1. Transfer 1 mL of A and B into separate sterile microfuge tubes; label A and B.
2. Pellet by spinning for 30 s at top speed in the microfuge.
3. Discard supernatant and resuspend pellet in 1 mL sterile water.
4. Repeat **steps 2** and **3** twice.
5. Resuspend cells in 1 mL sterile water and then for each strain make a separate 10-fold dilution using sterile water.
6. On the back of the Petri dish, draw three small circles and label the strains "A," "B," and "A + B."
7. Transfer 10 µL of the 10^{-1} dilution of strain A onto the agar in the center of the circles labeled A and A + B. Close the plate and set aside until the spots have dried in.
8. Transfer 10 µL of the 10^{-1} dilution of B onto the agar in the center of the circles labeled B and A + B (*see* **Note 15**).
9. Incubate for 3–4 d. Multiple colonies should develop in the circle labeled A + B. None should grow in the circles containing the separate strains (*see* **Note 16**).
10. Restreak the diploid colonies on a selective plate to single colonies to isolate a pure strain (*see* **Notes 17** and **18**).

3.5. Sporulation and Spore Isolation

Sporulation is normally accomplished by taking actively growing diploid cells and transferring them to a medium that discourages fermentation and is limited with respect to nitrogen. The presence of potassium ions is also desirable. Depending on the strain in question, the isolation of haploid spores from diploid cells ***(8,10)*** can be challenging. The two most important parameters are: the percentage of the diploid cells that undergo sporulation and the separation of the haploid spores from the diploid cells. There are a number of procedures available for the induction, and isolation, of haploid spores from diploid cells. A robust one is provided below.

1. Lightly inoculate the presporulation medium and grow at 25–30°C for 2 d (*see* **Note 19**).
2. Harvest the cells on a bench-top centrifuge (2000–3000*g* for 5 min) and wash by resuspending the cells in sterile water and reharvesting.
3. Resuspend the cells in 5 mL of sterile water, and transfer 0.3 mL into the sporulation medium (*see* **Note 20**).
4. Incubate with vigorous shaking at 25–30°C for 3–4 d (*see* **Note 21**).
5. Check under the microscope for the development of asci (*see* **Notes 22** and **23**).
6. Spore isolation is hereafter determined by the percentage of diploid cells that have produced four-spore asci. The isolation described below (**steps 7–19**) works reasonably well when the percentage sporulation is in excess of 30% (*see* **Note 24**).

7. Harvest and wash the sporulated cells as in **step 2**.
8. Resuspend the cells in 50 µL β-glucuronidase (*see* **Note 25**) in a microfuge tube and incubate at room temperature for 1 h.
9. Pellet using a 30-s spin in a microfuge.
10. Wash twice by resuspending in 1 mL sterile water and harvesting again (note size of pellet).
11. Resuspend in 500 µL of sterile water.
12. Add an equal volume of sterile mineral oil (*see* **Note 26**) and vortex vigorously for 30–60 s (*see* **Note 27**).
13. Separate the two phases by a 1- to 2-s spin at the lowest possible speed in the microfuge (*see* **Note 28**).
14. Transfer the top mineral oil layer into a fresh microfuge tube, add 500 µL of sterile water, vortex vigorously for 30–60 s, and repeat **step 13**.
15. Repeat **step 14**.
16. Transfer the top mineral oil layer into a fresh microfuge tube and concentrate the spores by spinning at top speed on the microfuge for 30–60 s (*see* **Note 29**).
17. Remove supernatant.
18. Resuspend by vigorous vortex mixing in 40–50 µL of sterile mineral oil.
19. Using a sterile glass spreader, vigorously spread 15 µL aliquots onto nonselective plates. Incubate for 2–3 d at 28–30°C.
20. Test 20 colonies for some of the complementary genetic markers carried by the haploid parents to check that there has been a good differential extraction of spores from the diploid cells (*see* **Note 30**).

3.6. Accessing Relevant Internet Resources

Everything that you wanted to know about the yeast *S. cerevisiae* but were afraid to ask can essentially be accessed through one main Web site: the *Saccharomyces* genome database (SGD) site curated at Stanford University (http://www.yeastgenome.org/). The entire curated genome is accessible from it, complete with links to papers written about each of the identified Open Reading Frames. It also provides links (http://www-deletion.stanford.edu/cgi-bin/deletion/search3.pl.) to enable workers to access strains carrying specifically deleted open reading frames. It even obviates the need for Northern blots in many incidences because, thanks to the availability of multiple microarray datasets, the expression level of each and every gene is available for a whole host of situations (http://db.yeastgenome.org/cgi-bin/SGD/expression/expressionConnection.pl), including heat stress, exposure to mating factor, sporulation, during the cell cycle, and expression during the diauxic shift. It even has a link to every laboratory in the world that researches this organism. It is sufficient to say that the SGD is an invaluable resource with links to many of relevant and exciting sites. An extraordinarily well-organized, user-friendly site, it is best appreciated and understood by being experienced at first-hand.

4. Notes

1. Strains harboring plasmids are best grown in media that lack the nutrient that selects for the auxotrophic marker (i.e., a strain harboring a plasmid with a *LEU2* wild-type gene should be grown in the absence of that amino acid).
2. A small number of some constituents are heat-sensitive (*see* **Tables 2** and **3**). These must be filter-sterilized and added to the medium after it has been autoclaved.
3. Liquid media can be stored at 4°C to prevent evaporation. However, if used regularly, it can be stored at room temperature.
4. Yeast cells can be prevented from undergoing fermentation and forced into the respiratory mode of growth by replacing the 2% (w/v) glucose in the medium with 3% (w/v) glycerol. Such media can be used to test the integrity of the mitochondrial respiratory chain, thereby identifying petite yeast strains that lack functional mitochondria.
5. Where the yeast strain requires growth under selective conditions (e.g., a strain carrying an unstable plasmid), the streaking out and patching should be completed on the appropriate selective medium.
6. It is good practice to check out all of the phenotypic markers using one of the patches from the second YEPD plate for strains newly acquired from other laboratories/suppliers. Clerical/storage errors frequently occur and a quick check ensures that the correct strain is being maintained.
7. It is good practice to return to the slopes each time an inoculum is required. Mutations can accumulate in strains if they are repeatedly subcultured from one experiment to the next.
8. Frozen stocks can also be maintained at –20°C, but should be subcultured every 5–6 yr.
9. Fresh slopes can be prepared annually using patches grown from the frozen stock.
10. Lyophilization can also be used to indefinitely maintain yeast strains.
11. Ideally one should allow a 10-fold difference between the volume of the media and the flask used to grow the cells (i.e., 100 mL in a 1-L flask). However, depending on the growth facilities and/or the number of cultures required in a given experiment, even these flask sizes can be impractical. Once cells are being shaken at a speed that is sufficient to prevent them falling to the bottom of the tube during overnight growth, they will grow quite happily. Here we suggest a fivefold difference in volume.
12. Different yeast strains grow to different sizes in different media. The OD_{600} measurement therefore will vary with respect to precise cell numbers. This measurement should therefore be standardized using a counting chamber or viable counts to determine exact cell numbers per OD unit.
13. It is good practice to plot a growth curve of each strain to accurately estimate division times.
14. Use dry (left on the bench for a few days) plates whenever possible. Freshly poured plates tend to be moist and it will take a lot longer for the spots to dry in.

15. Multiple matings can be set up on the same plate as long as the spots are well-separated.
16. A small number of small colonies occasionally can be found in the control circles owing to reversion, colonies on the mating spot should be much more numerous.
17. Diploid cells can be differentiated from haploids under the microscope because they are generally larger and have a different budding pattern: they bud from opposite poles of the cell, whereas haploid buds appear beside one another. A definitive diploid test is the ability to undergo sporulation to yield four-spored asci.
18. Matings can also be carried from haploid colonies growing on a plate by using sterile flat wooden applicator sticks to cross-streak the strains in question directly onto a rich plate. Diploids can be selected the following day by replica-plating to selective media using sterile velveteen pads.
19. Yeast strains that sporulate efficiently can be inoculated directly from actively growing YEPD cultures into sporulation medium without the presporulation step.
20. When a diploid cell has an auxotrophic requirement, it is best to provide it at 25% of the level of the appropriate supplement indicated in **Table 3**.
21. Although sporulation works best in liquid in our hands, the same procedure can be followed using solid media, add 2% agar to the recipes in **Table 4** and use a generous inoculum of cells when transferring to the sporulation medium.
22. If there are very few/no asci after 3–4 d reincubate for a further 3–4 d, checking for ascus development daily.
23. Poor sporulation can sometimes be alleviated by using different sporulation recipes and/or sporulating the strain at a lower temperature (15–20°C) for a longer period of time.
24. The procedure also works for lower percentage sporulation. The number of diploid cells making it through to the plating step can be minimized by repeating methods **step 14** and being prepared to screen more of the final colonies to identify the haploids. However, in our experience many diploids still get through.
25. β-glucuronidase works well, but any wall digesting enzyme preparation will do.
26. Sterile liquid paraffin can be used instead of mineral oil.
27. The hydrophobic spores preferentially partition into the hydrophobic liquid paraffin layer. The majority of diploid cells partition into the water.
28. The short spins aim to separate the phases without spinning the spores to the bottom of the tube; too fast and all the spores will end up discarded at the bottom of the tube!
29. If the extraction has worked, there should only be a small pellet of cells (compared to the pellet in **step 10** in methods). If the pellet is still big, repeat **steps 11–13**.
30. There is a good deal of "art" as opposed to "science" involved in this spore isolation procedure; one gets better with practice. Keep an eye on pellet size at each step to estimate how much differential extraction has occurred. Even then plated spores tend to stick together and colonies need to be restreaked and retested to ensure purity. While becoming familiar with this technique, it is a good idea to set up sporulating cultures on sequential days. That way the second culture is sporulating while the previously extracted spores are growing. Then if anything

goes wrong with the first attempt, material is immediately available for a second attempt.

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2

Isolation of Nucleic Acids

Michelle Hanna and Wei Xiao

Summary

Saccharomyces cerevisiae is an excellent model organism for the study of eukarotic genetics. Easy manipulation of yeast DNA is essential to its role in research, and studies of gene expression or regulation require analysis of RNA. This chapter presents quick and straightforward methods to isolate genomic DNA, plasmid DNA, or RNA from yeast. The isolation protocols presented here, which utilize a glass bead method to break through the cell wall, will yield plasmid DNA of sufficient quality to transform into *Escherichia coli*, genomic DNA that can be digested with restriction enzymes for Southern blotting, or RNA for use in applications such as Northern blots.

Key Words: Yeast; DNA isolation; plasmid isolation; RNA isolation; method; gene expression; gene regulation; glass bead method.

1. Introduction

The budding yeast *Saccharomyces cerevisiae* is often studied as a model eukaryotic organism. It is an excellent tool for genetic studies, both because of the ease of manipulation of the organism and the wealth of reference information available; Web sites such as the *Saccharomyces* Genome Database (SGD; http://www.yeastgenome.org) curated by Stanford University contain sequence information, database analysis tools, links to papers, and even links to researchers working in the area. An essential component of using yeast as a model for study is the ability to manipulate the genetic material. RNA is isolated for studies such as gene expression and regulation, for reverse transcriptase polymerase chain reaction, or for ribonuclease protection assays. DNA is isolated to confirm genotypes by Southern blotting or by sequencing, and plasmid DNA may be isolated to confirm that an observed phenotype is a result of a plasmid construct, to recover a plasmid for sequencing, or to transform into *Escherichia coli* for amplification.

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

One of the challenges of isolating nucleic acids from yeast cells is the cell wall. The two main methods of overcoming this barrier are first to create spheroplasts and isolate from them ***(1)***, or to use vortexing and glass beads to break through the cell wall ***(2)***. The methods presented here use the glass bead method; it is quick and straightforward, and eliminates the expense of using zymolyase. The DNA isolated by this method is suitable for restriction digest and Southern blots, or for transformation into *E. coli*.

The method used to isolate RNA is straightforward; the difficulty usually encountered in working with RNA is contamination by exogenous ribonucleases (RNases). Great care has to be taken to avoid this. Gloves must be worn at all times because RNases are present on the skin. Equipment or surfaces touched with bare hands are likely to be contaminated, and because RNases are quite stable this can be a persistent problem. Work areas, reagents, and tools must be specially prepared and protected. It is best to set aside a work area specifically for RNA work if possible so that it can be maintained as an RNase-free area. Separate bags of disposables such as tips and tubes can be kept in this area so that they are only opened with gloved hands. Disposables guaranteed to be RNase-free can be purchased. It is also best to have a separate set of chemicals set aside for RNA work. Even if not specified, unopened chemicals can be assumed to be RNase-free, but if chemicals have been used they may have been measured out with spatulas that previously have been handled without gloves, and thus the chemicals are likely to be contaminated.

2. Materials

2.1. DNA Extraction

1. DNA lysis buffer: 2% Triton X-100, 1% sodium dodecyl sulfate (SDS), 0.1 *M* NaCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA), pH 8.0, 10 m*M* Tris-HCl, pH 8.0.
2. Acid-washed glass beads: 0.4–0.5 mm glass beads, washed in hydrochloric acid then rinsed in copious amounts of water repeatedly until pH reaches 7.0. Beads are baked dry before use.
3. Phenol:chloroform:isoamyl alcohol (25:24:1): Phenol is prepared as per **ref. *3***. Phenol is corrosive, so gloves should be worn during its preparation and handling. Phenol is first melted at 68°C, then hydroxyquinilone is added to a final concentration of 0.1% (*see* **Note 1**). One volume of 0.5 *M* Tris-HCl, pH 8.0, is added and the solution is mixed vigorously. Once the two phases have separated, the upper phase is removed and discarded. One volume of 0.1 *M* Tris-HCl is added and mixed, and again the phases are allowed to separate and the upper phase is removed and discarded. This step is repeated until the phenol reaches a pH of 7.8 or higher (*see* **Note 2**). The phenol is then stored under 0.1 *M* Tris-HCl, pH 8.0, at 4°C.
4. 10 – 1 TE: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0.

5. RNase free of DNase: RNase stock is prepared as per **ref. *1***. RNase A is dissolved in 0.01 *M* sodium acetate, pH 5.2, at a concentration of 10 mg/mL. It is placed in a boiling water bath for 15 min to inactivate DNase, and allowed to cool to room temperature. One-tenth volume of 1 *M* Tris-HCl, pH 7.4, is added to adjust the pH (*see* **Note 3**). Aliquot and store at –20°C.

2.2. RNA Extraction

1. RNA lysis buffer: 0.5 *M* NaCl, 10 m*M* EDTA, 1% SDS, 0.2 *M* Tris-HCl, pH 7.6.
2. Acid-washed glass beads: prepared as in **Subheading 2.1.**
3. Phenol:chloroform:isoamyl alcohol (25:24:1): the phenol is prepared as in **Subheading 2.1.**
4. Diethylpyrocarbonate (DEPC)-water: 0.1% DEPC dissolved in distilled and deionized water, then autoclaved (*see* **Note 4**).

Wear gloves during the preparation of any reagents for use with RNA. Solutions must be prepared RNase-free: any glassware used should be baked overnight at 200°C; any reusable plastics should be soaked in DEPC water for at least 1 h and autoclaved; and DEPC water should be used in the making of solutions.

3. Methods

3.1. DNA Extraction (see *Fig. 1)*

1. Yeast cells may be cultured on plates or in liquid. It is best to use freshly grown cells. If the DNA to be isolated is genomic, cells may be grown in YPD. If you wish to isolate a plasmid, grow the cells in media that selects for the plasmid marker.
2. If cells were grown in liquid, collect cells in a screw-cap microcentrifuge tube (*see* **Note 5**) by centrifuging at 16,000*g* for 15 s and discarding the liquid. You may wish to repeat this step to collect additional cells, especially if the cells were grown in selective media. When you have collected enough cells, add 230 µL of DNA lysis buffer and resuspend the cell pellet. If cells were grown on a plate, collect cells with a sterile toothpick or loop, and resuspend them into 230 µL of DNA lysis buffer in a screw-cap microcentrifuge tube.
3. Add 0.4 g of acid-washed glass beads, and 200 µL phenol:chloroform:isoamyl alcohol. The phenol:chloroform mixture is hazardous, so gloves should be worn for any steps involving its use. Cap the tube, and make sure that it is tight enough that it will not leak during vortexing.
4. Vortex at top speed for 2 min if isolating plasmid DNA, and for 3 min if isolating genomic DNA (*see* **Note 6**).
5. Centrifuge at 16,000*g* for 5 min. Transfer aqueous phase (top layer) to a new microcentrifuge tube. Take care not to disturb the interface. Discard tubes of phenol:chloroform according to the requirements of your institution.
6. Add 600 µL of cold 95% ethanol (*see* **Note 7**) to precipitate the DNA, and keep the tube at –20°C for 30 min. Pellet the DNA by centrifugation at 16,000*g* for 15

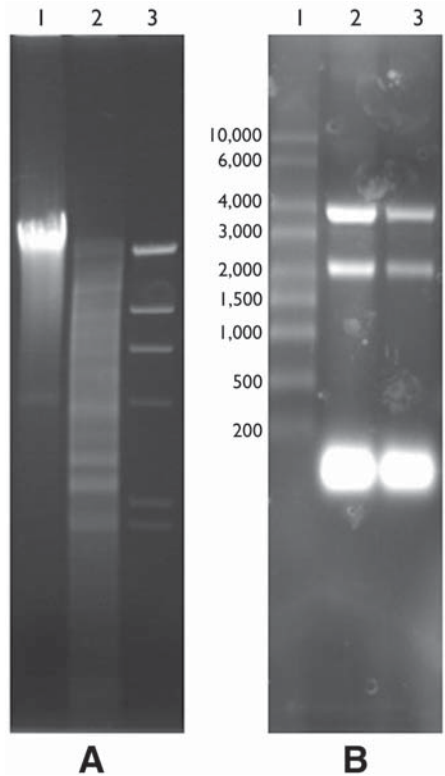

Fig. 1. (A) Yeast genomic DNA isolated by the protocol described. Lane 1, undigested DNA representing half of one isolated sample. Lane 2, the same quantity of DNA, digested with *Eco*R1. Lane 3, λ DNA digested with *Hin*dIII, used as a size marker. **(B)** Total yeast RNA isolated by the protocol described. Lane 1, RNA size marker with transcript sizes listed in base pairs (Transcript RNA Markers 0.2–10 kb, Sigma, St. Louis, MO). Lanes 2 and 3, 10 µL of isolated RNA (one-quarter of the isolated sample).

min, and discard ethanol. Tubes may be placed upside down to air-dry for 30 min, or may be dried under vacuum for a few minutes. If you are isolating plasmid DNA, this DNA pellet may be resuspended in water or TE and directly used to transform yeast or bacterial cells. If you are isolating genomic DNA, proceed to **step 7**.

7. Resuspend the DNA pellet in 200 µL of TE. Add 5 µL of RNase A, and incubate at 37°C for 10 min.
8. Add 8 µL of 5 *M* NaCl and 2 vol (about 430 µL) of cold 95% ethanol. Place at –20°C for 30 min. Pellet the DNA by centrifugation at 16,000*g* for 15 min, and discard ethanol. Tubes may be placed upside down to air-dry for 30 min, or may be dried under vacuum for a few minutes. Resuspend the DNA pellet in water or TE (*see* **Notes 8** and **9**).

3.2. RNA Extraction (see Fig. 1)

This protocol is for the isolation of total RNA, and is based on the protocol in **ref. *4***. There are other methods to isolate particular types of RNA, such as polyA RNA ***(5)***. Wear gloves throughout this protocol.

1. Culture yeast cells overnight at 30°C in 4 mL of the appropriate liquid medium.
2. Transfer culture to a 15-mL conical tube, and collect cells by centrifuging at 3000*g* for 4 min at room temperature. Discard the liquid medium, and add 2 mL of 0.1% DEPC water. Resuspend the cell pellet by vortexing briefly.
3. Centrifuge at 3000*g* for 4 min and discard the DEPC water.
4. Add 350 µL of Lysis Buffer and resuspend the cells. Transfer this mixture to a microcentrifuge tube (*see* **Note 5**), then add 0.4 g of acid-washed glass beads and 350 µL of phenol:chloroform:isoamyl alcohol. Vortex the tubes at top speed for approx 2.5 min.
5. Centrifuge the tubes at 16,000*g* for 4 min, then transfer the aqueous phase into a new microcentrifuge tube. Add 2.3 vol of 95% ethanol (about 0.8 mL; *see* **Note 10**). Mix well and centrifuge immediately at 16,000*g* for 4 min.
6. Discard the supernatant and wash the RNA pellet with 200 µL of 70% ethanol. Dry briefly under vacuum.
7. Dissolve the RNA pellet in 40 µL of DEPC water (*see* **Note 9**).

4. Notes

1. Hydroxyquinilone aids in inhibiting RNase, and it is both an antioxidant and a weak chelator of metal ions ***(6)***. It has the added benefit of coloring the phenol to make it readily distinguishable from the Tris buffer.
2. The final pH of the phenol is extremely important to the successful isolation of nucleic acids. If the phenol is at too acidic a pH, the nucleic acid will partition into the phenol and be discarded!
3. The pH must be adjusted after the boiling step, otherwise the RNase will precipitate during this step.
4. DEPC should be handled in a fume hood. The water should be allowed to incubate with the DEPC for at least 1 h (we usually leave it overnight) before autoclaving. DEPC in water will decompose into carbon dioxide and ethanol at room temperature with a chemical half-life of about 30 min. Any DEPC remaining after overnight incubation will be inactivated by autoclaving.
5. A screw-cap tube is recommended for this protocol rather than a snap-cap tube because while vortexing with phenol, a snap-cap tube is more likely to leak.
6. If you are isolating from several samples at once, a floater or other holder may be employed so that all tubes may be vortexed at once.
7. In our lab we routinely use 95% ethanol; however, 100% ethanol can be used instead.
8. The quantity of water or TE added depends on the number of cells isolated from and the desired final concentration of DNA. We usually use 25–50 µL.
9. A spectrophotometer may be used to determine DNA or RNA concentration in the isolated sample, if the sample is quite pure and sufficiently concentrated ***(3)***.

An optical density (OD) of 1 at 260 nm means that your sample contains approx 50 µg/mL of DNA, or approx 40 µg/mL of RNA. The ratio between readings at 260 nm and 280 nm is also important as an indicator of sample purity. Pure DNA should have an OD_{260}/OD_{280} value of 1.8, and a pure RNA sample should yield a value of 2.0. If the values are significantly lower, your sample is likely contaminated with proteins or phenol; in this case, spectrophotometric determination of the amount of DNA or RNA is not possible. If the sample is not pure enough or concentrated enough for spectrophotometric measurement, the amount of DNA or RNA can be estimated by gel electrophoresis of the sample alongside a sample of known concentration.

10. Various protocols call for a wide range of ethanol volumes, but the range of 2 to 2.5 vol is the most commonly used.

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3

Purification of Yeast Peroxisomes

Ben Distel and Astrid Kragt

Summary

Peroxisomes are ubiquitous subcellular organelles of eukaryotic cells. As with all organelles, peroxisomes can be purified from cell lysates using a combination of differential centrifugation and density gradient centrifugation. Here, we describe a method for purifying peroxisomes from the yeast *Saccharomyces cerevisiae*. The method involves gentle lysis of yeast spheroplasts, followed by differential centrifugation to obtain a crude organelle pellet enriched for peroxisomes and mitochondria. To separate peroxisomes from mitochondria, the organelle pellet is resuspended and spun through a sucrose density gradient. Peroxisomes purified in this way can be used to explore whether a protein of interest might be associated with the organelle.

Key Words: *Saccharomyces cerevisiae*; peroxisome; purification; differential centrifugation; density gradient centrifugation.

1. Introduction

The group of De Duve first described the isolation of peroxisomes from rat liver tissue. These organelles can be separated from other subcellular organelles, such as lysosomes and mitochondria, because of their relatively high equilibrium density in sucrose (~1.24 g/cm^3). The isolation of peroxisomes from the yeast *Saccharomyces cerevisiae*, however, has been hampered by the fact that, under standard growth conditions, peroxisomes are present in low numbers. Also, the lability of peroxisomes in general has complicated their purification.

Two observations have greatly facilitiated the isolation of peroxisomes from yeast: first, peroxisomes are induced by growth on a fatty acid ***(1)***, and second, peroxisomes are more stable at low pH (~5.5). The method making use of these two findings (adapted from a procedure described by Goodman ***[2]***), involves osmotic lysis of yeast spheroplasts at low pH, followed by differential cen-

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

trifugation to obtain an organelle pellet. After resuspension, the pellet is layered on a discontinuous sucrose gradient to separate mitochondria and peroxisomes. Peroxisomes purified in this way are relatively stable and show only minor contamination with mitochondria.

2. Materials

Prepare all solutions in distilled water, unless otherwise indicated.

1. 2.4 *M* sorbitol (sterilized in an autoclave, stable at 4°C for up to 1 mo).
2. 0.5 *M* 2[*N*-morpholino] ethanesulfonic acid (MES)/KOH, pH 5.5. (Stable at 20°C for up to 1 mo.)
3. 0.5 *M* potassium phosphate buffer, pH 7.5; mix 83 mL 0.5 *M* K_2HPO_4 with 17 mL 0.5 *M* K_2HPO_4.
4. 0.5 *M* Ethylenediaminetetraacetic acid (EDTA), pH 8.0.
5. 0.1 *M* Tris/H_2SO_4, pH 9.4.
6. Buffer A: 50 m*M* potassium phosphate, pH 7.5, 1 m*M* EDTA, 1 m*M* KC1.
7. Buffer B: 5 m*M* MES, pH 5.5, 1 m*M* EDTA, 1 m*M* KC1.
8. Buffer A plus 1.2 *M* sorbitol.
9. Buffer B plus 1.2 *M* sorbitol.
10. Buffer B plus 0.65 *M* sorbitol.
11. 1 *M* dithiothreitol (DTT) prepared in H_2O, store at –20°C.
12. 0.2 *M* phenylmethylsulfonyl fluoride (PMSF) in ethanol (store at –20°C; handle with care; toxic). PMSF is unstable in water and must be added just prior to use.
13. Zymolyase (100,000 U/g) is obtained from ICN Immunobiologicals (High Wycombe, UK).
14. WOYglu medium: Contains 0.67% (w/v) yeast nitrogen base without amino acids (WO; Difco, Surrey, UK; add separately from a 6.7% [w/v] stock solution sterilized by filtration), 0.1% (w/v) yeast extract, 0.3% (w/v) glucose, and amino acids (20 µg/mL; add separately from a 100X stock solution sterilized by filtration) as needed.
15. Induction medium: Contains 0.5% (w/v) bactopeptone, 0.3% (w/v) yeast extract, 0.12% (v/v) oleic acid, 0.2% (v/v) Tween-40, and 0.5% (w/v) KH_2PO_4 (adjusted to pH 6.0 with NaOH) sterilized by autoclaving.
16. Discontinuous sucrose gradients are prepared in quick-seal tubes (polyallomer, 25 × 89 mm, Beckman Instruments, Fullerton, CA). Each gradient consists of 5 mL 60% (w/w) sucrose, 12 mL 46% (w/w), 12 mL 44% (w/w), 5 mL 40% (w/w), all in buffer B (*see* **Note 1**).
17. Sucrose gradient overlay consists of 20% (w/w) sucrose in buffer B.

3. Methods

1. Grow a starter culture of a wild-type *S. cerevisiae* strain (e.g., BJ1991; American type culture collection, Rockville, MD; *see* **Note 2**) overnight in 10 mL of WOYglu medium in a 100-mL conical flask with vigorous aeration at 28°C.

2. Next morning, measure the cell density at 600 nm and inoculate a flask of WOYglu at an OD_{600} of 0.15 and grow to an OD_{600} of 1.0–1.5. Dilute the culture 1:100 in fresh WOYglu and incubate at 28°C overnight.
3. Repeat **step 2** (*see* **Note 3**). Next morning measure the cell density and inoculate 300 mL WOYglu (in 2-L flask) at an OD_{600} of 0.15. Grow to an OD_{600} of 1.0. Pellet the cells and resuspend in 10 mL of induction medium; use 5 mL to inoculate 1 L of induction medium. Incubate overnight at 28°C with vigorous aeration.
4. Harvest the cells at room temperature by centrifugation (4000*g*, 5 min) and wash twice with distilled water.
5. Determine the wet weight of the cell pellet and resuspend cells at 0.125 g/mL in 0.1 *M* Tris/H_2SO_4, pH 9.4, with 10 m*M* DTT (add fresh from 1 *M* stock solution). Incubate 20 to 30 min at 30°C with gentle shaking.
6. Harvest cells (as described in **step 4**) and resuspend in buffer A plus 1.2 *M* sorbitol. Centrifuge (4000*g*, 5 min).
7. Resuspend cells to 0.125 g/mL in buffer A plus 1.2 *M* sorbitol. Add zymolyase (100,000 U/g) to a final concentration of 1 mg enzyme/g of cells. Incubate 20–60 min at 30°C with gentle agitation to convert the cells to spheroplasts.
8. Check osmotic fragility as follows: Gently mix one drop of cell suspension with an equal volume H_2O and observe under the microscope at ×1000 magnification. Look for swollen spheroplasts. Add an extra drop of H_2O to lyse the spheroplasts and look for remnants of cell wall material. When most of the cell wall material has disappeared, the conversion to spheroplasts is complete.
9. All subsequent steps are performed at 4°C. Harvest spheroplasts (4000*g*, 5 min) and carefully resuspend the pellet in buffer B (*see* **Note 4**) plus 1.2 *M* sorbitol. Avoid shearing of spheroplasts (*see* **Note 5**). Recentrifuge and wash twice in the same buffer.
10. Resuspend the spheroplasts (the same volume as **step 7**) in buffer B plus 0.65 *M* sorbitol and add PMSF to a final concentration of 1 m*M*. Monitor lysis microscopically. We generally observe lysis of >80% of the spheroplasts. If lysis is not complete, gently shear spheroplasts using a Dounce homogenizer.
11. Centrifuge the homogenate (2000*g*, 10 min), save the supernatant, and resuspend the pellet in buffer B plus 0.65 *M* sorbitol. Spin (2000*g*, 10 min), pool the supernatant with that of the previous centrifugation step, and centrifuge (2000*g*, 10 min) (*see* **Note 6**).
12. Take the pooled "low-speed" supernatant and centrifuge 30 min at 20,000g.
13. Discard supernatant and carefully resuspend organelle pellet in 30% (w/w) sucrose in buffer B to a concentration of about 5 mg of protein/mL. Avoid shearing of the organelles.
14. Apply 2 mL to each 39 mL discontinuous sucrose gradient in a quick-seal tube. Fill up the tube with 20% (w/w) sucrose in buffer B and seal.
15. Centrifuge 2.5 h in a vertical rotor (Beckman VTi 50 or equivalent) at 34,500g_{av} (*see* **Note 7**).
16. Two bands will be visible in the gradient, a broad band at the 46 to 44% sucrose interphase containing the mitochondria and a smaller band at the 60 to 46% inter-

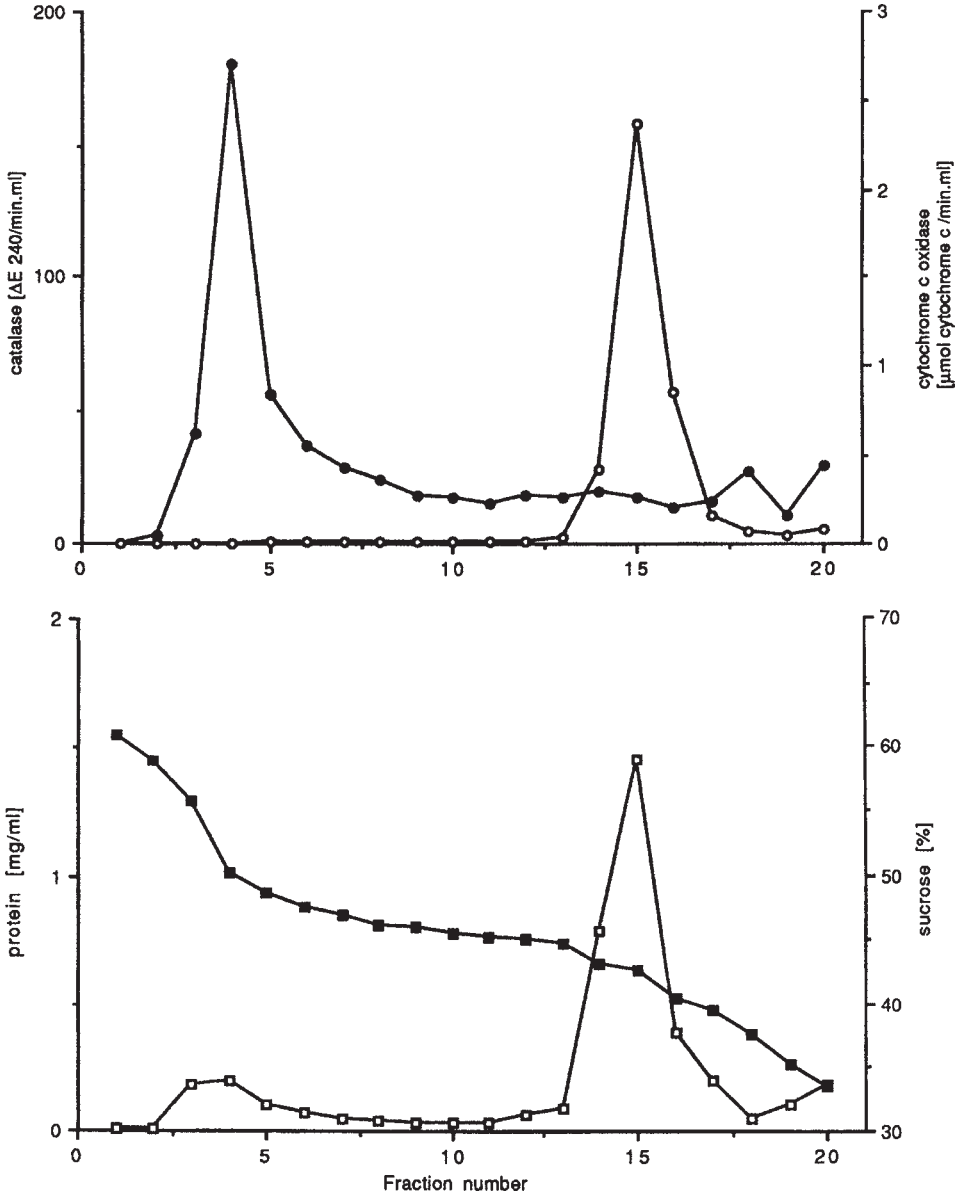

Fig. 1. Distribution of mitochondrial and peroxisomal enzymes after discontinuous sucrose gradient centrifugation of a 20,000*g* subcellular pellet. (■) sucrose, (□) protein, (●) catalase, (○) cytochrome *c* oxidase. Fractionation is from bottom (fraction 1) to top (fraction 20) of the gradient.

phase containing the peroxisomes (*see* **Note 8**). Gradients can be analyzed by collecting 2-mL fractions and measuring catalase (***3***; a peroxisomal marker) and cytochrome *c* oxidase (***4***; a mitochondrial marker). A typical enzyme profile is given in **Fig. 1** (*see* **Note 9**).

4. Notes

1. Note that the sucrose solutions are (w/w) and *not* (w/v).
2. We routinely use the *S. cerevisiae* strain BJ1991 for isolation of peroxisomes; however, other strains may work as well. Before using other strains, check for peroxisome induction on oleate containing medium by measuring β-oxidation enzymes (***5***) or by inspection of thin sections under the electron microscope.
3. To get optimal induction of peroxisomes, cells must rapidly divide prior to the shift to oleate medium. This is accomplished by extensive preculturing of the cells on glucose for 3 d. However, in induction medium the glucose concentration must be kept low because peroxisome proliferation in yeast is repressed by glucose. The best results are obtained when cells are harvested 12–18 h after the shift to induction medium.
4. Digestion of the yeast cell wall with zymolyase is optimal at pH 7.5. However, yeast peroxisomes are unstable at this pH and must be isolated at pH 5.5–6.0. Therefore, all steps following the zymolyase treatment are performed at pH 5.5.
5. Try to avoid shearing of spheroplasts (or peroxisomes) while resuspending pellets. Never use pipets with a narrow tip; use a paint brush or glass rod to resuspend pellets.
6. In the first low-speed pellet, a considerable proportion of the organelles is trapped in aggregated structures. Although resuspension of the pellet releases some of the organelles, a large part cannot be further fractionated. We (and others) have not been able to solve this problem.
7. The advantage of using a vertical rotor is that separation of the organelles is achieved within 3 h. This makes it possible to complete the isolation procedure within 1 d. A longer run in a swingout rotor is in principle also possible. However, we do not recommend this since peroxisomes are relatively labile organelles.
8. Peroxisomes can be harvested from the sucrose gradients by puncturing the tubes at the bottom with a wide-gage needle and collecting the 60–46% sucrose interphase. Sucrose fractions can be stored at –80°C after rapid freezing in liquid nitrogen without severe damage of the organelles. However, they should be thawed only once and used immediately.
9. Only a fraction of the peroxisomes is recovered in the final sucrose density gradient owing to the high losses in the low-speed pellet (*see* **Note 6**). Contamination of the peroxisomal peak fractions with mitochondrial protein is low, however. Therefore, this fractionation procedure is suitable for the assignment of enzymatic activities or proteins to peroxisomes.
10. Alternatively, peroxisomes can be purified on continuous 15 to 36% Nycodenz gradients with a cushion of 42% Nycodenz, dissolved in 5 m*M* MES, pH 6.0, 1 m*M*

KC1 and 8.5% sucrose ***(6)***. Note that when applying a Nycodenz gradient, the organellar pellet of **step 12** should be resuspended in 0.65 *M* sorbitol in buffer B.

11. A recent example of a study of yeast peroxisome biology is that by Bottger et al. ***(7)***.

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4

Isolation of Yeast Plasma Membranes

Barry Panaretou and Peter Piper

Summary

The plasma membrane is dynamic, with both its lipid and protein composition changing to facilitate adaptation to the ambient conditions. Biochemical activities to pre-existing proteins will also change. To monitor these variations, the cell membrane must be isolated. Moreover, the preparations must be free of contamination from the variety of other membranes in the cell, principally those associated with the golgi, endoplasmic reticulum (ER), the nucleus, and the vacuole. We describe a method for isolating plasma membranes that avoids incubation with enzymes that degrade the cell wall, thereby avoiding physiological changes that may lead to alteration in profile and activity of membrane proteins as well as avoiding changes that may alter lipid composition. We have used this method to show that, in response to heat shock, the plasma membrane acquires a novel heat-shock protein (HSP) and displays a decline in the levels of the abundant H^+ translocating ATPase.

Key Words: Plasma membrane; discontinuous density centrifugation; plasma membrane H^+ ATPase.

1. Introduction

Significant progress has been made recently in functional analysis of plasma membrane proteins, much of which has been at the level of procedures that localize the protein. Originally, total cellular membranes would be separated along sucrose gradients and an assay would be performed for the protein under investigation. The biochemical activity would be compared to that of marker enzymes of the various organellar membranes and this would serve as a method of localizing the protein. This has been superceded by methods involving immunocytochemial localization by epitope-tagging the corresponding gene, typically with the HA tag, or tagging with green fluorescent protein (GFP) (*see* **Note 1**). Frequently this would be accompanied by co-localization against proteins known to be resident at specific cellular locations. These analyses can be

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

extended by assaying biochemical activity of the protein or even monitoring levels of the protein *per se*, necessitating purification of plasma membranes. Straightforward determination of levels of a plasma membrane protein simply by measurements using crude lysates or total cell membrane preparations can be misleading, because the most important consideration is the amount of correctly localized protein. For example, functioning vacuolar ATPase (V-ATPase) is required for efficient targeting of Pma1, the plasma membrane H^+ ATPase. Total levels of Pma1 remain largely unchanged in cells mutant for V-ATPase, but Pma1 accumulates in the endoplasmic reticulum (ER), and the amount of Pma1 in the plasma membrane is largely reduced ***(1)***. If it is necessary to quantify levels of a plasma membrane protein along the secretory pathway, then assay of fractions along a sucrose gradient can be performed, as described in Sorin et al. ***(2)***.

Plasma membranes can be prepared from yeast by initially spheroplasting the cells ***(3)***. These procedures give high yields. However, an extended incubation (at least 30–45 min at 30°C) with zymolyase is required in order to remove the cell walls. This will almost certainly cause physiological changes, which may be reflected in alterations to plasma membrane components and will certainly influence biochemical activities of plasma membrane proteins as well as the MAP kinase cascade that responds to cell integrity *per se* ***(4)***. Also, cells cannot be spheroplasted in certain physiological states, such as stationary phase. Spheroplasting was therefore considered unsuitable for our own studies on the effects of stress on the proteins of the *Saccharomyces cerevisiae* plasma membrane ***(5)***. The procedure described in the Methods section, involving rapidly disrupting cells by vortexing with glass beads, avoids this problem. It is our slightly modified version of the plasma membrane isolation of Serrano ***(6–8)***, in which membranes are banded on sucrose density gradients. The plasma membranes obtained are of high purity, and the procedure is ideally suited to comparative studies of the plasma membranes from cells of different physiological states. Using this method, we extracted plasma membranes from unstressed and heat- shocked cells. We identified two major changes in plasma membrane protein composition associated with heat shock; namely, the appearance of a heat-shock protein (Hsp30) and a significant reduction in the levels of the H^+ translocating ATPase encoded by *PMA1* (*see* **Fig. 1**).

2. Materials

1. Buffer A: 25 m*M* imidazole (adjusted to pH 7.0 with HCl). This buffer is stored at 4°C. The following protease inhibitors are added just before use (*see* **Note 2**):
   a. 2.5 µg/mL pepstatin A (Sigma, St. Louis, MO) from a 2.5 mg/mL stock in methanol, stored at –20°C.

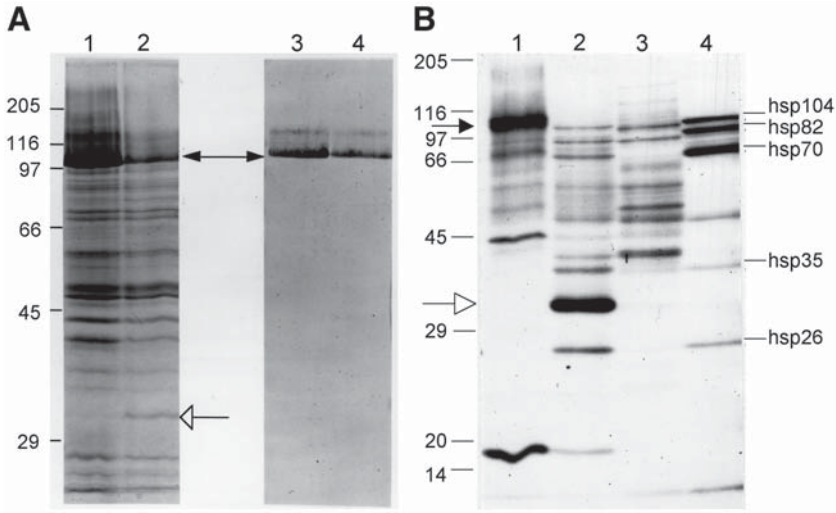

Fig. 1. (A) Proteins of isolated plasma membranes of unstressed and heat-shocked *S. cerevisiae*. A 12.5% SDS-PAGE gel stained with Coomassie blue, of plasma membrane proteins from (1) unstressed cells and (2) cells given a 40 min 20–40°C heat shock. Molecular masses (kDa) of markers are indicated on the left. On the right are indicated the 100 kDa plasma membrane H^+ ATPase (Pma1, solid arrow) and the Hsp30 from heat-shocked cells (open arrow). 3 and 4, a Western blot of the same samples (1 and 2, respectively) probed with antisera raised against Pma1. Each lane was loaded with the same amount of protein. **(B)** In vivo labeling of proteins of the plasma membrane and cytosol before and during heat shock using [^{3}H]-Leucine. Autoradiograph of a 15% SDS-PAGE gel. 1 and 2, correspond to the same samples described in (A); 3 and 4, cytosolic fractions from the unstressed and stressed cells, respectively. Molecular masses (kDa) are indicated on the left. Major heat-shock proteins are indicated on the right. Solid arrow, Pma1; open arrow, Hsp30 of heat-shocked plasma membranes. (Reproduced with permission from **ref. *5***.)

   b. 1 in 100 dilution of Complete EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals, Basel, Switzerland) from a stock in water (two tablets dissolved in 2 mL water). The stock can be stored at –20°C for up to 3 mo.
   c. EDTA itself can act as a protease inhibitor, but should be excluded because it may interfere with protein stability, biochemical assay, or affinity purification using cobalt/nickel chelates. If this is not a consideration, then ethylenediaminetetraacetic acid (EDTA) can be added to a final concentration of 2 m*M*.
2. Solutions of 0.4, 1.1, 1.65, and 2.25 *M* sucrose in buffer A, containing the protease inhibitors as in **step 1**.

3. Discontinuous sucrose gradients, prepared by overlaying three 4-mL layers of 2.25, 1.65, and 1.1 *M* sucrose in a 14 × 89 mm Beckman (Fullerton, CA) ultraclear tube.
4. Buffer B: 25 m*M* imidazole-HCl, pH 7.0, 50% (v/v) glycerol, containing the protease inhibitors as in **step 1**.
5. Glass beads with a diameter of 425–600 µ (Sigma). These have been acid-washed by the manufacturer and are ready to use. The 0.45-µm mesh beads manufactured by BDH (now part of VWR, Leicestershire, UK) are also suitable and less expensive, but these must be acid-washed prior to use, by soaking in HCl overnight followed by washing in water until the pH reaches 6.5. Care should be taken to ensure HCl does not come into contact with skin. All steps involving acid-washing must take place in a fume hood.

3. Methods

1. Harvest a 1 L culture by centrifugation (5 min, 5000*g*), then resuspend the cells in 80 mL 0.4 *M* sucrose in buffer A.
2. Divide the cell suspension between two 50-mL polycarbonate centrifuge tubes and pellet the cells (5000*g* for 10 min).
3. Add to the cell pellet two times the pellet volume of glass beads followed by just enough 0.4 *M* sucrose in buffer A to cover the cells and glass beads.
4. Vortex 2 min on a whirlimixer, then keep on ice for 30 s. Repeat this step two more times.
5. Dilute three times in 0.4 *M* sucrose in buffer A.
6. Centrifuge at 530*g* (2500 rpm in Sorvall SS34 rotor) for 20 min, to pellet unbroken cells and glass beads.
7. Recentrifuge the supernatant from **step 6** at 22,000*g* (16,000 rpm in Sorvall SS34 rotor) for 30 min to obtain a pellet that includes the plasma membranes and mitochondria.
8. Resuspend the pellet from **step 7** in 2 mL buffer A by gentle vortexing (30 s) (*see* **Note 3**).
9. Load 1-mL aliquots of resuspended membranes onto discontinuous sucrose gradients (*see* **Subheading 2.3.**) and centrifuge either overnight (14 h) at 80,000*g* (22,000 rpm) or 6 h at 284,000*g* (40,000 rpm) in the Beckmann SW41 or SW40Ti rotor.
10. Membranes banding at the 2.25/1.65 *M* sucrose interface are essentially pure plasma membranes ***(7,8)***, although a smaller proportion of the plasma membranes corresponding to about one-third of the plasma membrane ATPase activity bands together with mitochondria at the 1.65/1.10 *M* sucrose interface ***(7,8)***. Collect the membranes at these interfaces from the top of the gradient with a Pasteur pipet, dilute four times with buffer A, pellet at 30,000*g* (18,000 rpm Beckman 50 Ti rotor) for 40 min, resuspend in buffer B, and store at –20°C. Purity of membranes can then be assessed (*see* **Notes 4** and **5**).

4. Notes

1. If a membrane protein is to be localized using an epitope or GFP tag, yeast cells that carry the fusion construct as the only gene copy must be used. Membrane proteins are channeled through the secretory apparatus, and higher levels than

those in wild-type cells may overload this pathway leading to bottlenecks in the ER or golgi. An example of genomic epitope-tagging of the multidrug transporter Tpo1, via integration of a polymerase chain reaction (PCR)-generated cassette, is described by Albertsen et al. ***(9)***.

2. The method involves a long centrifugation step, and slight contamination with proteases could cause problems. We found this to be the case when isolating plasma membranes from heat-shocked cells. Also, high vacuolar protease activity is associated with nutritional stress, which could cause problems if plasma membrane proteins were to be analyzed from starved cells ***(10)***. Use of the protease inhibitor cocktail in buffer A should overcome these problems. If significant protein degradation is noticed, it will be owing to release of proteases from the vacuole, and is usually an artefact associated with disruption of cells. To overcome this, *PEP4* can be deleted from the strains being investigated, by targeted gene deletion (described elsewhere in this volume). *PEP4* is selected not simply because it is a protease itself, but because it is required for the in vivo activation of a number of vacuolar zymogens ***(11)***.
3. Resuspension in smaller volumes is not advisable, because it will cause the membranes to band as solid discs that are difficult to remove from the tubes during **steps 9** and **10**.
4. One of the best ways to assess the purity of yeast plasma membranes is to assay the fraction of the ATPase activity subject to orthovanadate inhibition ***(7)***. The plasma membrane ATPase is inhibited by orthovanadate.
5. Yields from this method tend to be low, but the membranes obtained are of high purity. If high yields are required, spheroplasted cells can be disrupted and membranes can be isolated via entrapment by dense cationic silica beads, as described by Chaney et al. ***(3)***. It should be noted that treatment with enzymes that degrade the cell wall could change biochemical properties of membrane proteins as well as affect levels of the proteins themselves.

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5

Isolation of Yeast Mitochondria

Chris Meisinger, Nikolaus Pfanner, and Kaye N. Truscott

Summary

Often preparations of isolated organelles contain other, unwanted, cellular components. For biochemical experiments to determine the localization of newly identified proteins, or to determine the whole set of proteins (or the proteome) from a desired organelle, these unwanted components often confuse the resulting data. For these types of studies, it is crucial to have highly pure fractions of the desired organelle. Here we describe a protocol for purification of mitochondria from *Saccharomyces cerevisiae* cells devoid of contamination from other cellular compartments.

Key Words: Mitochondria; *Saccharomyces cerevisiae*; organelle; proteome; protein import.

1. Introduction

Owing to the ease of genetic manipulation, the yeast *Saccharomyces cerevisiae* is an ideal organism for the study of many basic cellular mechanisms in eukaryotic cells. Their organelles can be rapidly enriched in sufficient quantities for the analysis of specific functions such as metabolite or protein transport.

We describe here procedures for the isolation of both crude and highly pure yeast mitochondria. The contents of yeast cells are made accessible by a combination of enzymatic digestion of the cell wall and physical disruption of the resulting spheroplasts. Owing to the variable density of the cellular contents, differential centrifugation is employed to isolate an enriched mitochondrial fraction in a matter of hours. This method is derived from protocols published by Daum et al. ***(1)*** and Hartl et al. ***(2)***. At this stage mitochondria are sufficiently pure for use in specific *in organello* assays such as preprotein import into mitochondria ***(3)***. The mitochondria may also be of sufficient purity to use as the starting material for isolation of mitochondrial proteins or protein com-

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

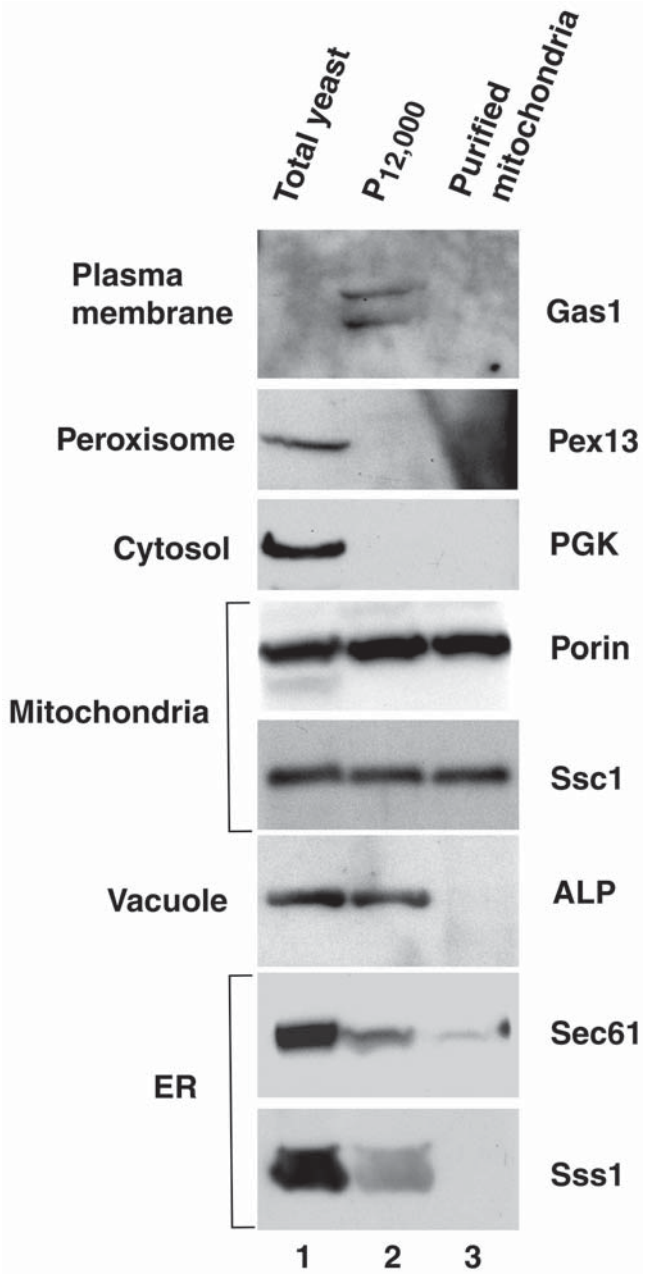

Fig. 1. Western-blot analysis of crude mitochondrial fraction (pellet of the 12,000g spin, $P_{12,000}$, lane 2) and mitochondria purified via sucrose gradient (lane 3). Samples were separated onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on polyvinylidine difluoride (PVDF) membrane followed by immunodecoration with specific antisera directed against the indicated marker

plexes ***(4)***. This crude preparation of mitochondria, however, is contaminated with vacuoles, endoplasmic reticulum (ER), and plasma membrane (*see* **Fig. 1**, lane 2) and is therefore unsuitable for such purposes as defining the cellular localization of newly identified proteins. Consequently we describe a protocol for the isolation of highly enriched mitochondria, the purity of which is illustrated in **Fig. 1** (lane 3). This additional purification step is achieved using sucrose density centrifugation and adds approx 2 h to the total procedure ***(5)***. We have found that the purity of mitochondria isolated from yeast cells grown on glycerol using sucrose gradients as described here is greater than that obtained using Nycodenz gradients as published by Glick and Pon ***(6)***, where yeast cells were grown on lactate. Both crude and highly pure preparations of mitochondria can be stored frozen without loss of functional integrity, at least with respect to protein transport ***(5)***. Further separation of isolated mitochondria into subcompartments such as the outer membrane or intermembrane space is possible, but this is described elsewhere ***(7,8)***. The purification method described here was recently applied successfully for the analysis of the complete yeast mitochondrial proteome ***(9)***.

2. Materials

All solutions should be made in distilled water. Equipment and solutions for yeast culture should be sterile.

1. DTT Buffer: 100 m*M* Tris-H_2SO_4, pH 9.4, 10 m*M* dithiothreitol (DTT), prewarmed to 30°C. Add DTT prior to use. A 1 *M* Tris-H_2SO_4 stock can be stored at room temperature.
2. Zymolyase Buffer: 1.2 *M* sorbitol, 20 m*M* potassium phosphate, pH 7.4.
3. Zymolyase-20T (Seikagaku Kogyo Co., Tokyo, Japan).
4. Homogenization Buffer: 0.6 *M* sorbitol, 10 m*M* Tris-HCl, pH 7.4, 1 m*M* ethylenediaminetetraacetic acid (EDTA), 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.2% (w/v) bovine serum albumin (BSA; essentially fatty acid-free, Sigma-Aldrich, Taufkirchen, Germany). Add PMSF from a freshly prepared 100 m*M* stock in ethanol just prior to use. Pre-cool homogenization buffer to 4°C.
5. SEM Buffer: 250 m*M* sucrose, 1 m*M* EDTA, 10 m*M* MOPS-KOH, pH 7.2. Store SEM buffer at 4°C for up to 1 mo. Precool SEM buffer to 4°C.
6. EM Buffer: 1 m*M* EDTA, 10 m*M* MOPS-KOH, pH 7.2. For sucrose step gradients, prepare stocks of 60%/32%/23% and 15% (w/v) sucrose in EM and store in the fridge (stable up to 1 mo).

Fig. 1. *(continued)* proteins. Equal volume aliquots were loaded to show the relative ratio of mitochondrial fraction compared to total yeast. (Gas1, glycophospholipid-anchored surface protein; Pex13, peroxin-13; PGK, phosphoglycerol kinase; Ssc1, mitochondrial heat shock protein (Hsp) 70; ALP, alkaline phosphatase; Sec61, component of ER protein translocase; Sss1, component of ER protein translocase.)

7. Preparative ultracentrifuge and a swinging bucket rotor. We use a Beckman SW41 Ti rotor and Beckman Ultra-Clear™ Centrifuge Tubes, 14 ×89 mm.
8. Culture medium (autoclaved): YPG (for growth on nonfermentable carbon source): 1% (w/v) yeast extract (Difco), 2% (w/v) Bactopeptone (Difco), 3% (v/v) glycerol, pH 5.0 (HCl). YPD (for growth on fermentable carbon source): 1% yeast extract; 2% Bactopeptone, 2% glucose, pH 5.0 (HCl).

3. Methods

3.1. Growth of Yeast Cells

1. Prepare a pre-culture by inoculating 100 mL of media (YPG) in a 400-mL flask with yeast cells from a plate and incubate overnight at 30°C with vigorous shaking.
2. Dilute cells from the preculture into 1.5 L of YPG media in 5-L flasks to an OD_{600nm} of 0.03 (*see* **Note 1**). Incubate the culture at 30°C with vigorous shaking to ensure sufficient aeration until an OD_{600nm} of 2 is reached. For wild-type yeast strain YPH499 ***(10)***, which has a typical doubling time of 4 h in YPG, this will take around 28 h (*see* **Note 2**).

3.2. Isolation of Crude Mitochondrial Fraction

1. Pellet the cells at room temperature by centrifugation at 3000*g* for 5 min and wash with distilled water. Collect cells in 1–2 centrifuge pots by centrifugation as above, pour off the water, then determine the weight of the cells. A typical yield is about 3–4 g/L culture (OD 2).
2. Resuspend the yeast pellet in prewarmed DTT buffer (2 mL/g wet weight cells) and shake slowly (approx 80 rpm) at 30°C for 20 min.
3. Centrifuge at 3000*g* for 5 min and resuspend the pellet in Zymolyase buffer (about 7 mL/g wet weight as previously determined [**step 1**]).
4. Centrifuge as in **step 3** and resuspend pellet in Zymolyase buffer (7 mL/g wet weight) containing 3 mg Zymolyase per gram wet weight. Shake slowly at 30°C for 30–45 min (*see* **Notes 3** and **4**).
5. Harvest the cells by centrifugation at 3000*g* for 5 min and wash pellet with Zymolyase buffer (7 mL/g wet weight).
6. Centrifuge at 3000*g* for 5 min and resuspend pellet in ice-cold homogenization buffer (6.5 mL/g wet weight). The lysed material must be maintained at low temperature to avoid proteolysis; therefore, pre-cool buffers and equipment such as rotors to 4°C. From this step onwards, always work on ice between centrifugation steps, which are performed at 4°C (*see* **Note 5**).
7. With appropriate volumes (depending on the size of the homogenizer) homogenize the spheroplasts with 15 strokes using a glass-Teflon homogenizer. When homogenization is complete, dilute the sample twofold with homogenization buffer.
8. Centrifuge the homogenate at 1500*g* for 5 min to pellet cell debris and nuclei. Collect the supernatants.
9. Centrifuge the supernatant at 4000*g* for 5 min. Discard the pellet.

10. Isolate the mitochondrial fraction by centrifugation of the supernatant from **step 9** at 12,000*g* for 15 min (*see* **Note 6**).
11. Gently resuspend the crude mitochondrial pellet in SEM (*see* **Note 7**). Determine the protein concentration, then dilute the sample to a final concentration of 5–10 mg/mL protein with ice-cold SEM. The sample is flash-frozen in liquid nitrogen and stored at –80°C. Import competence is not affected even after storage for more than 1 yr. To avoid repeated freeze thawing of mitochondrial samples to be used in functional assays such as preprotein import, the mitochondrial suspension is aliquoted into small volumes of 25–50 µL. For further purification via sucrose gradients (*see* **Subheading 3.3.**), keep the nonaliquoted fractions at a concentration of 5 mg/mL on ice (*see* **Note 8**).

3.3. Isolation of Highly Purified Mitochondria

1. Prepare sucrose step gradients: Load 1.5 mL 60% sucrose/EM onto the bottom of the centrifuge tube. Pipet carefully, without disturbing the phases, stepwise 4 mL 32%, 1.5 mL 23%, and 1.5 mL 15% sucrose/EM. Keep the tubes in the fridge.
2. Adjust the crude mitochondrial fraction to a concentration of 5 mg/mL with SEM and treat them with 10 strokes in a glass-Teflon potter.
3. Carefully load the homogenate (0.2–1 mL) on top of the sucrose gradient and centrifuge for 1 h at 2°C and 134,000*g*. Turn off the centrifuge breaking system.
4. Recover the purified mitochondria with a Pasteur pipet from the 60%/32% sucrose interface (*see* **Note 9**).
5. Pool all mitochondrial samples, then dilute with 2 vol of SEM. Pellet the mitochondria using 10,000*g* at 2°C. Resuspend the mitochondrial pellet in SEM, then adjust the protein concentration to 5–10 mg/mL with SEM.
6. For storage, *see* **Subheading 3.2., step 11**.

4. Notes

1. A typical scale of yeast culture in our lab is 9 L (6 × 1.5 L), which finally yields about 80 mg mitochondrial protein.
2. Doubling times vary with yeast strains, media, and the temperature of incubation. Therefore, the doubling time of a particular strain under defined growth conditions should be determined before setting up the culture.
3. If you are working with a temperature-sensitive mutant strain, we recommend that zymolyase treatment also be performed at a lower temperature; i.e., when permissive growth of the mutant is at 24°C, then also perform zymolyase treatment at 24°C.
4. To check the level of cell-wall degradation by zymolyase, add 50 µL of the yeast suspension, prior to and following zymolyase treatment, to separate glass test tubes each containing 2 mL of water. After 30–45 min incubation, the zymolyase-treated solution should be clear (owing to osmotic disruption of spheroplasts), whereas the nontreated solution should still be turbid. Alternatively, measure the OD_{600nm} of each solution. The absorbance ratio of nontreated to treated yeast suspensions should be at least 3:1. If the ratio is lower, add more zymolyase and incubate for a further 15 min.

5. BSA can be omitted from the homogenization buffer if it causes a problem for purification of proteins later on.
6. A slight improvement in the purity of the crude mitochondrial fraction can be achieved when the pellet from the 12,000*g* centrifugation (*see* **Subheading 3.2., step 10**) is resuspended in SEM and again centrifuged at 4000*g*. A second 12,000*g* spin of the supernatant recovers the mitochondrial fraction.
7. If you use a yellow tip for resuspension, cut off about 2 mm of the tip to avoid disruption of the mitochondria.
8. When purifying mitochondria with sucrose gradients, the crude mitochondrial fraction can be frozen beforehand in large aliquots. Just thaw them on ice before running the gradients.
9. About 80% of the protein from the loaded crude mitochondrial fraction is typically recovered in the 60%/32% interface. Most contaminants and some residual mitochondria are retained in the 15% and 23% phases.

Acknowledgments

We thank Dr. R. Taylor for discussion and critical comments, and Drs. H. Riezman, T. Sommer, T. H. Stevens, and W. Kunau for antisera.

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6

Extraction of Yeast Lipids

Roger Schneiter and Günther Daum

Summary

Quantitative extraction of lipids from the tissue or microorganism of choice is key to their subsequent analysis. In this chapter, we describe a simple and rapid protocol that relies on glass bead disruption in the presence of organic solvents to quantitatively extract lipids from yeast cells.

Key Words: Lipids; phospholipids; fatty acids; yeast; thin-layer chromatography.

1. Introduction

The aim of the present chapter is to consider practical aspects of the isolation of lipids from yeast cells and from isolated subcellular fractions. Because lipids are water-insoluble, their extraction requires a combination of polar and nonpolar organic solvents. The goal of the extraction procedure generally is a quantitative recovery of all the different lipid classes.

Three methods for the liquid–liquid extraction of lipids widely cited in the literature are those of Folch, Lees, and Stanley ***(1)***; Bligh and Dyer ***(2)***; and Ways and Hanahan ***(3)***. All three methods use chloroform/methanol (2:1, v/v) as the extracting solvent. These protocols can be adapted for the extraction of lipids from whole yeast cells by including a step to break open the yeast cell wall, which is usually done by disintegrating the cells in the presence of glass beads. Glass beads can be omitted for the extraction of lipids from isolated subcellular fractions.

The Folch et al. procedure ***(1)*** described herein is the most common method used. It employs 20 volumes of chloroform/methanol (2:1; v/v) per volume of tissue or membrane pellet to yield a single homogenous suspension. If the ratio is smaller (12–15 volumes), two phases will be formed. The single phase of solvent, however, provides a better interaction of polar and nonpolar solvents

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

with membrane lipids. A higher ratio is probably harmless to some extent, but too high a ratio of solvent will lower the water content, making polar lipid extraction incomplete.

Lipid extracts have the tendency to trap water-soluble, nonlipid material, such as sugars, amino acids, and salts, within lipid micelles. These can be removed simply by washing the extract with 0.2 volume of water or various salt solutions. Addition of the wash medium results in the formation of a two-phase system: the lower phase will consist of chloroform/methanol/water (86:14:1; per vol.) while the upper phase will contain the same solvents in a ratio of 3:48:47 (per vol.). The lower phase, which comprises approx two-thirds of the total volume, contains the lipids. The upper phase, which retains the nonlipid contaminants, is discarded.

After the lipid extract has been washed to remove any water-soluble, nonlipid components, the organic phase is evaporated off by using either a rotary vapor or, in case the volume is small, by drying under a stream of nitrogen.

Taken together, the preparation of a lipid extract includes the following basic steps:

1. Homogenization of the cells in the presence of organic solvents and glass beads.
2. Extraction of the lipids with chloroform/methanol (2:1; v/v).
3. Removal of nonlipid contaminants by washing the extract with aqueous salt solutions.
4. Drying of the extract by removal of the organic solvent.

2. Materials

2.1. Isolation of Whole Cell Lipids

1. Medium for growing yeast (e.g., YEPD): 1% (w/v) yeast extract, 1% (w/v) bactopeptone, 2% (w/v) glucose.
2. Glass beads: 0.25–0.3 mm diameter (e.g., Braun Melsungen, Melsungen, Germany).
3. Merkenschlager (Braun Melsungen) cell homogenizer with fitting glass bottles.
4. The organic solvent used, i.e., chloroform and methanol, are of analytical grade. All solvent manipulations are to be carried out in a fume cupboard while wearing protective clothing, because chloroform is listed as harmful, and methanol is toxic.
5. Sintered glass funnel.
6. Wash solutions: 0.034% $MgCl_2$; 2 *N* KCl/methanol (4:1; v/v); artificial upper phase: chloroform/methanol/water (3:48:47; per vol.).
7. Table-top centrifuge with appropriate glass tubes.
8. Rotary evaporator.
9. 12-mL Pyrex glass vials with Teflon liner caps.

3. Methods

3.1. Isolation of Whole Cell Lipids

1. Grow yeast in a suitable medium (e.g., YEPD) so as to obtain 50–200 mg dry cell weight (0.5–2 g wet weight); cell densities between 5×10^6 and 2×10^8 cell/mL, in 100 mL medium, should be adequate.
2. Harvest cells by centrifugation at 300*g* for 10 min. Pour off supernatant and discard.
3. Wash the cells with deionized water.
4. Mix the harvested cells (1.5 mL aqueous suspension) with 10 mL methanol and transfer to a 70-mL Merckenschlager glass bottle.
5. Add 20 g (15 mL) of glass beads and disrupt the cells at 1700 rpm in a Merckenschlager under CO_2 cooling by shaking for four periods of 30 s with 30-s cooling intervals.
6. Add 20 mL chloroform to the suspension to give a ratio of chloroform/methanol of 2:1 (v/v) and stir the suspension for 1 h on a flat-bed stirrer at room temperature.
7. Filter the extract through a sintered glass funnel and wash the glass beads with 10 mL chloroform/methanol (2:1; v/v).
8. Transfer the extract to a 250-mL glass beaker, add 10 mL (0.2 volume) of 0.034% $MgCl_2$ solution, and stir for 10 min.
9. Centrifuge the extract in appropriate glass vials at 3000 rpm in a table-top centrifuge for 5 min.
10. Aspirate off the upper aqueous layer and wash the organic phase with 10 mL of 2 *N* KCl/methanol (4:1; v/v).
11. Centrifuge again at 3000 rpm in a table-top centrifuge for 5 min.
12. Aspirate off the upper aqueous layer and wash the organic phase with 10 mL of artificial upper phase (chloroform/methanol/water; 3:48:47; per vol.).
13. Centrifuge again at 3000 rpm in a table-top centrifuge for 5 min.
14. Aspirate off the upper aqueous layer, including the protein layer that formed at the phase boundary, and repeatedly wash the organic phase with 10 mL of the artificial upper phase until the phase boundary becomes clear.
15. Transfer the organic phase to a round-bottom flask and evaporate the solvent in a rotary evaporator set to 55°C and 200 mBar.
16. Dissolve the lipid film in 5–6 mL chloroform/methanol (2:1, v/v) and store the lipid extract in a high-quality glass vial (e.g., Pyrex) tightly sealed with Teflon liner caps at –20°C. This solution is now ready for lipid analysis and quantitative estimations of different lipid classes. It contains approx 0.1–1.0 mg lipids/mL and is composed mainly of phospholipids, sterols, steryl esters, and triacylglycerols.

4. Notes

1. The organic solvents used are toxic or even carcinogenic. Chloroform/methanol mixtures will rapidly leach the skin lipids from hands. Further contact with the solvents will give rise to irritation. It is therefore advisable to handle all solvents with care. If mixtures of chloroform and methanol spill over a part of the body, the body part should immediately be kept under cold running water to minimize the burning sensation, which could last for about 10 min.
2. Owing to the large volume of organic solvent used for the extraction any nonvolatile trace contaminants in these solvents will become enriched in the final lipid extract and may then interfere with subsequent characterization, e.g., by gas liquid chromatography (GLC) analysis. To avoid this, the purest possible solvents (preferably redistilled) should be used.
3. Plastic bottles or tips should not be used at any stage of the extraction procedure. They contain oxidants and low molecular-weight polymers, which will be dissolved by the solvents and which might subsequently interfere with the lipid analysis. Therefore, glass materials should be used throughout the procedure.
4. To extract the lipids from isolated membrane fraction (15–20 mg protein), the extraction procedure can be downscaled accordingly. In this case, glass beads are not required for the extraction step.
5. The evaporation of the organic solvent should be accomplished at as low a temperature as possible. If residual water does not azeotrope off in the first evaporation, it may be necessary to add further portions of chloroform to the evaporation flask and to repeat the evaporation step.
6. The merits and disadvantages of the Folch method have been discussed in detail in the literature (for a comprehensive review, *see* **ref. *4***). The method gives excellent recoveries for neutral lipids and diacylglycerophospholipids (95–99%), whereas lysophospholipids are only partly recovered. The efficiency of the washing procedure depends on the presence of salts, which alter the distribution of lipids and practically eliminate them from the upper phase. In the absence of salts, substantial amounts of acidic lipids are present in the aqueous phase and would be lost during washing.
   It is possible to depart from the procedure, but it is essential that, while washing the extracts, the ratio of chloroform/methanol/water is 8:4:3 (per vol). In calculating these proportions, it is important to remember that the extract contains all the water from the cells, which in the original protocol by Folch et al. ***(1)*** is assumed to have a density of 1.0 g/L. In the modified Folch method described here, the tissue is assumed to have a density of approx 1.0 g/mL.
7. When large amounts of material have to be extracted and quantitative recovery of the different lipid classes is not essential, the method of Bligh and Dyer ***(2)*** is preferable to the Folch procedure. Major advantages of this method are that much smaller amounts of solvent are required and that less nonlipid material is retained in the extract. The quantities of chloroform/methanol (2:1; v/v) are again such that when mixed with the water in the tissue, a single-phase solution is formed.

8. For a quantitative recovery of the very polar yeast sphingolipids, the use of a specialized extraction procedure developed by Hanson and Lester ***(5)*** is recommended.
9. Because most lipids are typical surface-active substances, their extraction is frequently accompanied by the formation of stable emulsions. Unfortunately no general recommendations for avoiding emulsion formation can be given. Usually the best way to break down emulsions is centrifugation, which mostly results in complete phase separation. Phase separation frequently is accompanied by formation of an intermediate fluffy layer. In such cases, the upper phase is removed by suction, a small amount of the upper phase of the starting Folch system is added, and the intermediate layer is washed by rotating the vessel. After separation of the phases, the upper layer is again removed by suction and the whole operation is repeated several times. Finally, chloroform/methanol (2:1; v/v) is added until the intermediate phase disappears. Any solid material remaining is then removed by filtration.

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7

Two-Dimensional Gel Electrophoresis of Total Yeast Proteins

Hélian Boucherie and Christelle Monribot-Espagne

Summary

Two-dimensional gel electrophoresis (2-DE) offers the opportunity of separating several hundred proteins from a total yeast cellular extract. A detailed description is provided here of the different steps required for the separation and visualization of radiolabeled yeast proteins on high-resolution (24 cm × 20 cm) 2-D gels. Two methods of protein separation are described. They essentially differ by the way proteins are separated in the first dimension. One is based on the use of isoelectric focusing (IEF) gels (carrier ampholytes) and the other on the use of ready-made IPG gels (immobilines). These methods allow separating soluble proteins from a total yeast cellular extract with an isoelectric point ranging between pH 4.0 and 7.0 and a molecular weight ranging between 15,000 and 150,000.

Key Words: Two-dimensional gel electrophoresis; IEF; IPG; carrier ampholytes; immobilines; yeast proteome; radiolabeling.

1. Introduction

Two-dimensional gel electrophoresis (2-DE), which was originally described by O'Farrell ***(1)***, separates proteins in the first dimension according to their isoelectric point, and in the second dimension according to their molecular weight. It offers the opportunity of separating several hundred proteins from a cellular extract. For a long time, this technique has been reputed to be difficult. During the last few years, the quality of chemicals for 2-DE applications has been improved and the different steps of the technique have been optimized. In addition, commercialized equipments specifically designed for 2-DE have been made available. These developments make now 2-DE a technique accessible to any laboratory. This is particularly true for yeast laboratories because yeast is a rather favorable organism for 2-DE investigation: the

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

number of protein species is limited to a few thousand (6000) and there is little posttranslational modification. In combination with the methods of protein identification based on mass spectrometry, this technique has become an invaluable tool for quantitative and qualitative studies on the yeast proteome.

Two 2-DE methods of protein separation are described here. They essentially differ by the way proteins are separated in the first dimension. One depends on the use of carrier ampholytes and the other on the use of "Immobilines." In the first case, the pH gradient is generated by an electric field to an acrylamide gel containing a mixture of free carrier ampholytes (isoelectric focusing [IEF]). In the second case, the pH gradient is generated by "Immobilines," which are co-polymerized with the acrylamide matrix (immobilized pH gradient gel electrophoresis [IPGE]). Each method has advantages and drawbacks. The use of carrier ampholytes reduces the number of steps for protein separation and requires loading smaller amounts of protein. On the other hand, ready-made first-dimensional gels for IPGE are commercially available. The use of these gels is more favorable to inter-laboratory comparison of 2-D protein patterns.

The proteins separated by these 2-DE methods are visualized by radiolabeling after incorporation of radioactive amino acids into proteins. Radiolabeling has three major interests for protein detection: (1) it is the most sensitive way to detect proteins; (2) combined with the storage phosphor screen technology, it is possible to obtain quantitative data on protein spots with a linear dynamic range of four orders of magnitude; (3) because proteins are labeled in vivo, radiolabeling raises the possibility of distinguishing between protein synthesis (pulse labeling) and protein accumulation (long-term labeling), a possibility not offered by post-separation staining procedures. Procedures for protein visualization based on the use of dyes, fluorescent molecules, or silver staining can be used also. They have been widely described elsewhere ***(2)***.

We have limited the description of the procedures to techniques that separate yeast proteins with an isoelectric point between pH 4.0 and 7.0 and a molecular weight between 15,000 and 150,000. These techniques combine a wide description of the yeast proteome and a good protein resolution. It is possible to extend the proteomic view to basic proteins by using IPG gels with a broader pH range (from 3.0–10.0 or 3.0–11.0; **refs. *3*** and ***4***). However in this case the resolution power is reduced. It is also possible to use narrow immobilized pH gradients gels in order to carry more detailed research ***(5)***. Finally, IPG gels specifically devised to the separation of basic proteins are also available ***(6)***. Recent examples of the application of 2-DE to the study of the yeast proteome is provided by our investigation on the regulatory mechanisms controlling changes in protein synthesis during the diauxic shift ***(7,8)***.

The 2-D analysis of radiolabeled proteins involves four basic steps: protein labeling, sample preparation, 2-D protein separation, and protein visualization. Each of these steps is covered separately in the following sections.

2. Materials

See **Note 1** for general comments on the Materials section.

2.1. Cell Culture and Radiolabeling

1. Culture medium: for radiolabeling, yeast strains are grown in a minimum medium devoid of the amino acid used for the labeling. Usually we use YNB medium (0.67 % [w/v] yeast nitrogen base without amino acids, 2% [w/v] glucose, 85 m*M* succinate/NaOH, pH 5.8) supplemented with tyrosine 24 µg/mL (*see* **Note 2**). Amino acids and bases are added as required if using auxotrophic strains. Cultures are performed are 22°C.
2. Radiolabeled amino acids: we generally use L-[^{35}S]-methionine (specific radioactivity >1000 Ci/mmol, 10 µCi/µL) (*see* **Note 3**). L-[U-^{14}C]-leucine (>300 mCi/mmol) can be used also.
3. L-[^{35}S]-methionine labeling solution. The labeling solution is obtained by mixing pure L-[^{35}S]-methionine with appropriate amounts of 1.25 10^{-5} *M* unlabeled methionine solution. For labeling proteins that will be separated by IEF migration, prepare L-[^{35}S]-methionine with a specific radioactivity of 500 Ci/mmol and a radioactive concentration of 5.6 µCi/µL. For proteins that will be separated by IPG, the labeling solution is 250 Ci/mmol and 3 µCi/µL. Store as single use aliquots, 55 µL and 105 µL, respectively, at –80°C.

2.2. Sample Preparation

1. Acid-washed glass beads (0.45-mm diameter, Braun Biotech, Bethelhem, PA).
2. Sample Buffer A: 0.1 *M* Tris-HCl, pH 8.0, 0.66% (w/v) Triton X-100 (PlusOne, Amersham Bioscience, Uppsala, Sweden). Filter through 0.45-µm pore filter and store as aliquots (500 µL) at –20°C.
3. RNAse A solution: 0.05 *M* $MgCl_2$, 200 Kunitz U/mL RNAse A (type XII-A from bovine pancreas; Sigma, St. Louis, MO), 0.5 *M* Tris-HCl, pH 7.0. Aliquots (25 µL) are stored at –20°C.
4. DNAse I (from bovine pancreas, 10,000 U/mL, Amersham Bioscience) stored at –20°C.
5. Sample Buffer B: 21.4% (w/v) CHAPS (Sigma), 0.85% (w/v) Pharmalytes 3–10 (Amersham Bioscience). Aliquots (150 µL) are stored at –20°C.
6. 1 *M* dithiothreitol (DTT) solution (Fluka Sigma). Aliquots (20 µL) are stored at –20°C.
7. Urea (PlusOne, Amersham Bioscience) and thiourea (Fluka Sigma).
8. Protein Assay Kit (Bio-Rad, Hercules, CA).
9. Liquid scintillation: Ready Value (Beckman Coulter, Fullerton, CA).
10. Microfiber filters (GF/C, Whatman, Maidstone, UK).

11. Equipment: Lyophilizer, MiniBeadBeater (Biospec Products, Bartlesville, OK), β counter.

2.3. Two-Dimensional Gel Electrophoresis Using IEF Gels

2.3.1. First Dimension

1. IEF acrylamide stock solution: prepare a solution 28.38% (w/v) acrylamide, 1.4% (w/v) *N*,*N*'-methylenebisacrylamide (both: PlusOne, Amersham Bioscience). The solution is filtered through a 0.45-µm pore filter, and kept at 4°C in a brown bottle to protect from light. Store for no more than 1 mo. This is a neurotoxin when unpolymerized, so care should be taken not to receive exposure.
2. 15% (w/v) CHAPS solution (Fluka Sigma). Filter through a 0.45-µm pore filter. Store at 4°C.
3. Ampholytes: Pharmalytes 3–10, 5–6, and 5–8 (Amersham Bioscience) stored at 4°C.
4. Urea (PlusOne, Amersham Bioscience).
5. 10% (w/v) ammonium persulfate solution (APS solution, PlusOne, Amersham Bioscience), prepared just before use.
6. Cathodic solution (0.1 *M* NaOH). To prepare 1 L of cathodic solution, dissolve 4 g NaOH in 1 L deionized water. De-aerate under vacuum for 15 min while continuously stirring. The cathodic solution is prepared just before use.
7. Anodic Solution (0.08 *M* H_3PO_4). To prepare 1 L of anodic solution dissolve 5.5 mL of 85% phosphoric acid in 1 L of deionized water. Prepare just before use.
8. Sample Buffer C solution: 32 m*M* Tris-HCl, pH 8.0, 0.21% (w/v) Triton X-100, 24 m*M* DTT, 7.5 *M* urea, 2.05 *M* thiourea, 3.76 % CHAPS, 0.15% Pharmalytes 3–10.
9. Overlay solution: 2.37 *M* urea, 2% (w/v) CHAPS, 0.5% Pharmalytes 3–10.
10. Equipment: Glass tubes 26 cm long, inner diameter 1 mm; electrophoresis apparatus with an upper and a lower chamber, that allows using 26 cm long gel tubes (Investigator 1-D running gels, Genomic Solutions, Chelmsford, MA; or Model 175 tube gel cell, Bio-Rad for example); power supply capable of up to 2000 V.

2.3.2. Second Dimension

1. 40% (w/v) acrylamide solution (acrylamide PAGE 40% solution, Amersham Bioscience) and 2% (w/v) *N*,*N*'-methylenebisacrylamide solution (PlusOne, Amersham Bioscience). Both solutions are kept at 4°C in the dark for no more than 1 mo after opening.
2. Slab gel buffer: 1.5 *M* Tris-HCl, pH 8.5 (130 g/L Trizma base, 66.3 g/L Trizma hydrochloride).
3. 10% (w/v) ammonium persulfate solution. Prepared just before use.
4. Running buffer: 192 m*M* glycine (for electrophoresis, Fluka Sigma), 25 m*M* Trizma Base, 0.2% (w/v) sodium dodecyl sulfate (SDS; Merck, White House Station, NJ).

5. Bromophenol blue: traces of Bromophenol in 1.5 mL of 50% glycerol solution (kept at –20°C).
6. Fixing solution 1: 50% (v/v) ethanol, 7.5% (v/v) acetic acid in distilled water.
7. Fixing solution 2: 25% (v/v) ethanol, 2.5% (v/v) acetic acid in distilled water.
8. Equipment: any commercialized equipment that allows vertical migration on gels that are 25 cm large, 20 cm long, and 1 mm thick; power supply; gel dryer.

2.4. Two-Dimensional Gel Electrophoresis Using IPG Gels

2.4.1. First Dimension

1. IPG gels are ready-made gels (Immobiline DryStrips pH 4.0–7.0, 24 cm long, Amersham Bioscience).
2. IPG buffer 4.0–7.0 (Amersham Bioscience).
3. Rehydration buffer: 7 *M* urea, 2 *M* thiourea, 2.5% CHAPS, 0.4% IPG buffer 4.0–7.0, 0.2% Triton X-100, 20 m*M* DTT. Prepare just before use.
4. Pefabloc SC 20 m*M* solution (Pefabloc SC Plus; Roche, Indianapolis, IN) stored in 100-µL aliquots at –20°C.
5. PSC-Protector solution (Pefabloc SC Plus, Roche) stored at 4°C.
6. Equilibration buffer: 6 *M* urea, 30% (w/v) glycerol, 2% (w/v) SDS, 0.05 *M* Tris-HCl, pH 6.8. Prepare just before use.
7. Prepare six small electrode wicks per strip (3MM paper, Whatman, 0.5 × 1 cm), plus one large cathodic wick and one large anodic wick (**Fig. 1A,B**).
8. Mineral oil: DryStrip Cover Fluid (PlusOne, Amersham Bioscience).
9. Equipment: Immobiline DryStrip Reswelling Tray 7–24 cm (Amersham Bioscience) and IEF Protean Cell with a 24-cm focusing tray (Bio-Rad). Thermal Printer DPU-414 (Bio-Rad).

2.4.2. Second Dimension

1. The solutions for preparing the slab gels are the same as in **Subheading 2.3.2.** In addition, you will need an Agarose solution: 1% agarose, 1% SDS, 5 m*M* Tris-HCl, pH 6.8 (keep at 4°C as 2-mL aliquots).
2. Running buffers: cathodic solution is 384 m*M* glycine, 25 m*M* Trizma base, 0.2% (w/v) SDS (*see* **Note 4**). The anodic solution is 192 m*M* glycine, 25 m*M* Trizma base, 0.15% (w/v) SDS. Prepare the solutions in deionized water just before use.
3. Fixing solution 1: 50% (v/v) ethanol, 7.5% (v/v) acetic acid in distilled water.
4. Fixing solution 2: 25% (v/v) ethanol, 2.5% (v/v) acetic acid in distilled water.
5. Equipment: same as in **Subheading 2.3.2.**

2.5. Visualization of Radioactive Protein

1. 3MM paper.
2. Saran wrap.
3. Equipment: slab gel dryer, phosphor screens, storage phosphor imaging system.

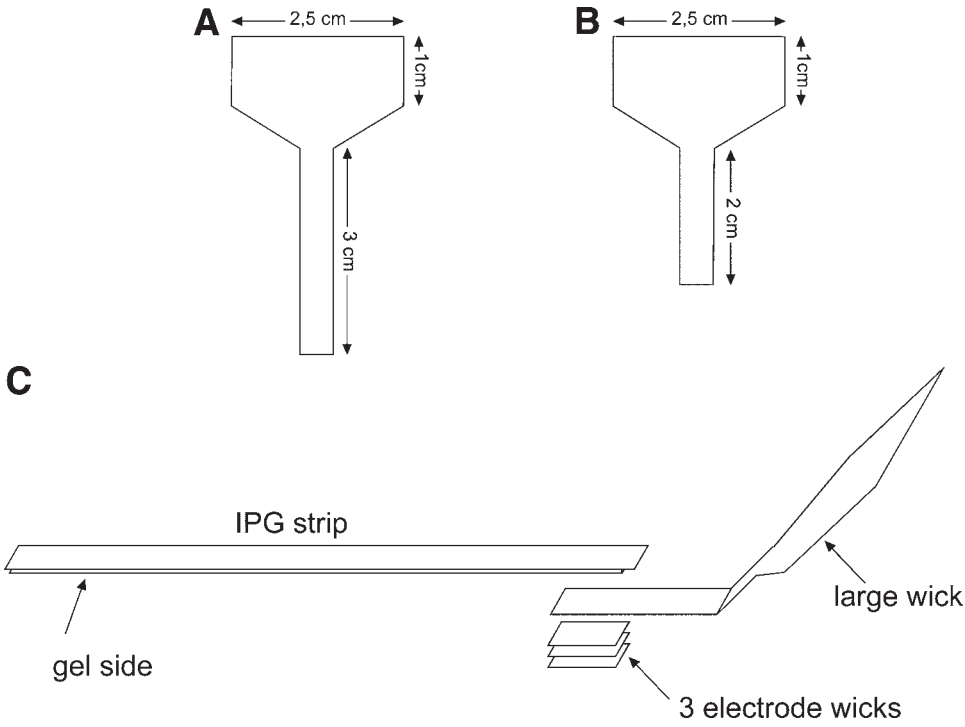

Fig. 1. Electrode wicks and their installation. **(A)** cathodic wick, **(B)** anodic wick, **(C)** positioning of the wicks.

3. Methods

The 2-DE techniques described here mainly differ by the first dimension, which, in one case, is based on the use of laboratory-made IEF gels and, in the other case, on the use of ready-made IPG gels. Cell culture, radiolabeling of proteins, sample preparation, the second dimension step of 2-DE, and visualization of separated proteins are basically the same.

3.1. Cell Culture and Radiolabeling

1. Cultures are performed at 22°C in 500-mL Erlenmeyer flasks containing 50 mL of medium. Cultures are shaken at 360 rpm and growth is monitored at 600 nm.
2. Labeling of exponentially growing cells is performed by labeling cells when the culture reaches an optical density (OD) of 1 (corresponding to 2.7×10^7 cells/mL for haploid strains).
3. At this point of the culture, a sample (1 mL for IEF, 2 mL for IPG) is transferred to a 20-mL sterile plastic tube sealed with a cotton plug and cells are labeled for 10 min with 300 µCi of [^{35}S]-methionine labeling solution (*see* **Note 5**).

4. After labeling, transfer the sample into a 2-mL microcentrifuge tube previously kept on ice and spin down the cells for 1 min at 9500*g*.
5. Rinse the pellet twice with the starting volume of ice-cold deionized water.
6. Resuspend the cells in 400 µL of ice-cold deionized water and transfer into a 500-µL microcentrifuge tube.
7. Spin down the cells at 9500*g* for 1 min.
8. Remove the supernatant and resuspend the cells in 30 µL of cold deionized water (*see* **Note 6**).
9. Keep the cells frozen at –80°C prior to being lyophilized.

3.2. Sample Preparation

Sample preparation is of particular importance as the quality of the final protein pattern is highly dependent on the quality of the protein sample. In particular, it is of importance to avoid protein degradation that would result in artefactual spots on the 2-D pattern. In the procedure described here, proteins are extracted by vigorously breaking cells with glass beads. Cell breakage is performed on lyophilized cells in the absence of buffer in order to minimize protein degradation. This sample preparation is a three-step procedure: cell lyophilization, cell breakage, and protein solubilization.

1. Make a hole in the cap of the microcentrifuge tube.
2. Put the microcentrifuge tube in a freeze-dry flask ended with a filter paper (to avoid radioactive contamination of the lyophilizer).
3. Lyophilize the cells for no more than 4 h (*see* **Note 7**).
4. Once lyophilization is done, remove the pierced cap and put on a new one.

At this stage, lyophilized cells can be kept at –80°C or immediately used for protein extraction.

5. Add 50 mg of glass beads.
6. Disrupt cells by shaking lyophilized cells in the presence of glass beads on a MiniBeadBeater. The tubes are agitated five times for 20 s, at 20-s intervals, leaving on ice between bursts of shaking.

At this stage, broken cells can be kept at –80°C or immediately used for protein solubilization.

7. Solubilize proteins by adding successively 120 µL of Sample Buffer A, 12 µL of RNAse solution, and 2 µL of DNAse, all previously kept at 4°C. Briefly vortex the sample.
8. Incubate for 1 min at 4°C.
9. Add 9 µL of 1 *M* DTT. Briefly vortex.
10. Add 161.1 mg of urea and 58.5 mg of thiourea.
11. Add 66 µL of Sample Buffer B. Mix gently moving the tube upside down several times.

12. After 5 min at room temperature, make sure that all urea is solubilized and centrifuge 4 min at 11,340*g*. Retain the supernatant, and store in aliquots (30 µL for IEF, 100 µL for IPG) at –80°C. Before freezing, keep 15 µL for counting the radioactivity and determining protein concentration (*see* **Note 8**).
13. Two microliters of sample are spotted on a microfiber filter for counting the radioactivity. Let filters dry at room temperature for 15 min. Soak filters twice for 10 min in 5% (w/v) TCA containing 1 g/L of methionine. Then place the dried filters in a counting vial, add 5 mL of liquid scintillation, and count in a β counter.
14. Ten microliters are used for determining protein concentration with the Bio-Rad Protein Assay.

3.3. Two-Dimensional Gel Electrophoresis Using IEF Gels

3.3.1. First Dimension With IEF Gels

3.3.1.1. Preparation of Isoelectric Focusing Gels

The first-dimensional gels are prepared the day before isoelectric focusing. The recipe is for 12 gels.

1. Keep the glass tubes for at least 1 h at 26°C.
2. Prepare the first-dimensional acrylamide gel solution in a corex tube by adding the components in the following order:
   a. 4 g of urea.
   b. 0.85 mL of IEF acrylamide stock solution.
   c. 1.73 mL of 15% CHAPS solution.
   d. 265 µL of Pharmalyte 3-10.
   e. 132 µL of Pharmalyte 5-6.
   f. 265 µL of Pharmalyte 5-8.
   g. 0.69 mL of deionized water. Dissolve urea by gentle mixing.
3. Warm the urea solution by keeping the corex tube in the palm of the hand. Do not heat the urea solution!
4. De-aerate the solution under vacuum for 3 min.
5. Initiate polymerization by adding 20 µL of 10% (w/v) APS, freshly prepared (*see* **Note 9**).
6. Swirl gently the corex tube by hand. Take care not to reintroduce oxygen into the solution.
7. Fill the glass tubes and leave the gels to polymerize overnight at 26°C (*see* **Note 10**).

3.3.1.2. Running Isoelectric Focusing Gels

The prefocusing and the focusing are carried out in an incubator maintained at 26°C.

1. De-aerate the cathodic solution under vacuum for at least 15 min.
2. Place the IEF tubes into the electrophoresis stand. Fill the lower chamber with the anodic solution. Remove air bubbles trapped at the bottom end of the tubes.

3. Overlay the gels with 20 µL of Sample Buffer C solution.
4. Fill up the tubes with the cathodic solution.
5. Fill the upper chamber with the cathodic solution, taking care not to disturb the sample buffer layer on the top of the gels.
6. Prefocus the gels as follows:
   a. 500 V for 30 min.
   b. 1000 V for 45 min.
   c. 1500 V for 15 min.
7. Remove the upper electrode buffer and the sample buffer.
8. Load the protein sample (*see* **Note 11**). Cover with 15 µL of Overlay solution. Fill up the tubes and the upper chamber with fresh upper electrode buffer.
9. Focusing is carried out as follows:
   a. 500 V for 15 min.
   b. 1000 V for 45 min.
   c. 1600 V for 21 h.
10. After focusing, gels are immediately extruded from the glass tubes onto a piece of Parafilm (*see* **Note 12**). For this purpose, we use a 2.5-mL syringe filled with water and fitted with a yellow pipet tip. The tip is inserted on the top of the end of the glass tube and the gel is pushed out by pressure on the syringe. Gels can be kept at –80°C for several months.

3.3.2. Second Dimension With IEF Gels

3.3.2.1. Preparation of Second-Dimension Slab Gels

Polyacrylamide slab gels are prepared the day before (*see* **Note 13**). The following instructions assume the use of equipment that allows vertical migration.

1. Before preparation of the casting cassette, wash the glass plates with deionized water and carefully air-dry.
2. In a 500-mL vacuum flask add successively:
   a. 51 mL of slab gel buffer.
   b. 55.56 mL of acrylamide solution.
   c. 36.7 mL of bisacrylamide solution.
   d. 65.24 mL of deionized water.
   e. De-aerate under vacuum for 3 min.
   f. Add 1 mL of 10% APS, freshly prepared.
   g. Mix gently.
   h. Add 136 µL of TEMED.
   i. Transfer to a 500-mL beaker. Take care not to re-introduce oxygen into the solution.
   j. Fill the cassettes up to 2 mm from the top.
   k. Gently overlay the gel solution with deionized water.
   l. After polymerization (30 min), rinse the top of the gel with deionized water and cover with slab gel buffer diluted four times.

3.3.2.2. Running Two-Dimensional Gels

1. Allow the first dimensional gels to thaw on their piece of Parafilm.
2. Rinse the top of the slab gels with deionized water.
3. Transfer the first-dimensional gels without equilibration to the top of the slab gels by pushing the rod gel with a blunt-ended spatula between the glass plates (*see* **Note 14**). Be sure that no air bubbles are trapped between the IEF gels and the surface of the acrylamide slab gels.
4. Insert the cassettes into the electrophoresis apparatus.
5. Carefully fill the space of the cassette above the rod gels with electrophoresis buffer using a pipet. Take care not to displace the first-dimensional gels.
6. Fill the upper and lower chamber with electrophoresis buffer.
7. Take care that no air bubbles are trapped at the lower surface of the slab gel. If so, remove the bubbles by a stream of buffer from a bent needle connected to a 50-mL syringe.
8. Add a drop of Bromophenol blue at both ends of the first-dimensional gel.
9. Run the slab gels as follows:
   a. 1.5 W per gel for 5 min.
   b. 8.5 W per gel until the Bromophenol Blue tracking dye reaches the bottom of the gel. Take care that the dye does not go outside the gel in order to avoid radioactive contamination of the lower buffer. The running is about 6 h.
10. After running, open the cassette with a spatula. Cut the lower corner of the gel corresponding to the basic side of the first dimensional gel to indicate its orientation.
11. Either briefly rinse the gel with fixing solution 1 and dry before exposure to phosphor screen plates, or leave the gel in fixing solution 1 overnight and then allow the gel to re-swell to its original size for 2 h in fixing solution 2.

3.4. Two-Dimensional Gel Electrophoresis Using IPG Gels

3.4.1. First Dimension

3.4.1.1. In-Gel Rehydration of the Sample

1. Per strip mix:
   a. 720 µL of rehydration buffer.
   b. x µL of protein sample corresponding to 75–100 µg of protein.
   c. 90 µL of 20 m*M* Pefabloc.
   d. 22.5 µL of Protector.
   e. Make up to 900 µL with deionized water.
2. Fill as many reservoir slots of the re-swelling tray as there are IPG strips to run with 900 µL of the above solution.
3. Carefully remove the protective cover sheets from the IPG strips. Cut 3 mm at the cathodic end of the plastic support film.
4. Position the IPG strips gel-side-down into the tray channels (avoid trapping air bubbles).

5. Slide the protective lid onto the re-swelling tray.
6. Allow the strips to rehydrate overnight at 22°C.

3.4.1.2. Isoelectric Focusing

1. Per strip, wet six small electrode wicks, plus one large cathodic and one large anodic electrode wick, with fresh deionized water.
2. Insert three superimposed small electrode wicks on top of both cathode and anode electrode wires in each channel of the focusing tray that will contain a strip.
3. Place the large electrode wicks above the small wicks (**Fig. 1C**).
4. Place the rehydrated strips gel-side-down in the channels of the focusing tray.
5. Cover each strip with about 2 mL of mineral oil.
6. Place the lid on the focusing tray and the tray on the Peltier platform.
7. Apply the following running conditions (*see* **Note 15**):
   a. 100 V for 1 h.
   b. 300 V for 4 h.
   c. 1600 V for 12 h.
   d. 3000 V for 5 h.
   e. 10000 V for 8 h.
   Limit the amperage to 50 µA per strip and run the IEF at 20°C.
8. After focusing, the strips are rinsed with deionized water and either used for the second dimension or stored at –80°C in a piece of Parafilm.

3.4.2. Second Dimension With IPG Gels

3.4.2.1. Preparation of the Second-Dimension Slab Gels

The procedure is the same as for the second dimension with IEF gels except that the cassettes are filled with the acrylamide solution up to only 6 mm from the top of the cassette in order to allow loading the strip.

3.4.2.2. Running Two-Dimensional Gels

1. Allow the strip to thaw on its piece of Parafilm.
2. Equilibrate each strip twice for 12 min in 12 mL of equilibration buffer containing 120 mg of DTT at 22°C.
3. During equilibration, rinse the top of the slab gel with deionized water and than drain the excess liquid.
4. After equilibration, gel strips are blotted to remove the excess equilibration buffer.
5. Lay the cassette at 45° in order to facilitate the application of the strips.
6. Lay the strip on the inner side of one of the two glass plates, with the plastic side on the glass plate.
7. With a blunt-ended spatula, push the strip between the two glass plates, seating it carefully on the top of the slab gel. Be sure that no air bubbles are trapped between the strip and the surface of the acrylamide SDS gel.
8. Insert the cassette with the strip in the electrophoresis apparatus.

9. Fill the space between the gel side of the strip and the glass plate with hot agarose solution. Do not cover the strip with agarose.
10. Allow the agarose to solidify for 5 min; cover the agarose with upper electrophoresis buffer.
11. Fill the upper tank and the lower tank with the corresponding buffers.
12. Check for the presence of air bubbles trapped at the bottom of the slab gel. If so, remove the bubbles by a stream of buffer from a bent needle connected to a syringe.
13. Add Bromophenol blue at both ends of the strips.
14. Run the slab gels at room temperature as follows:
    a. 1.5 W per gel for 15 min.
    b. 8 W per gel until the Bromophenol tracking dye reaches the bottom of the gel.
15. After electrophoresis, harvest the gel and prepare it for radioactive protein visualization as in **Subheading 3.3.2.**

3.5. Visualization of Radioactive Proteins

Gels must be dried before exposure for detection of radioactive proteins.

1. Lay the gel on a piece of 3MM paper.
2. Cover the gel with a sheet of Saran wrap.
3. Place the gel with the paper side down.
4. Dry for 1 h at 70°C.
5. Expose the dried gels to phosphor screens for the appropriate period of time and scan the screens with a storage phosphor imaging system (*see* **Note 16**).

Typical 2-D patterns obtained by the two techniques described previously are shown in **Fig. 2**. The two patterns are similar enough that information obtained with one type of gel can be easily transferred to the other type of gel. The IEF-based method provides a slightly larger view of the yeast proteome because proteins with an isoelectric point between pH 6.5 to pH 7.0 are not separated on IPG gels.

Several annotated 2-D reference maps of yeast proteins with the location of identified proteins have been reported in the literature ***(4,6,9–11)***. These reference maps can be useful to help the reader in identifying spots on his own gels. This is particularly true of the reference map corresponding to the IEF-based 2-DE technique described here (410 spots identified; ***11***). This map is also accessible through the Web ***(12)***. As can be observed in **Fig. 3**, identifications on this reference map can be easily transferred to IPG/2-D gels**.**

The beginner should keep in mind that the major problem to avoid when running 2-D gels is the occurrence of artefactual spots (*see* **Note 17**).

4. Notes

1. General comments on the Materials section follow.

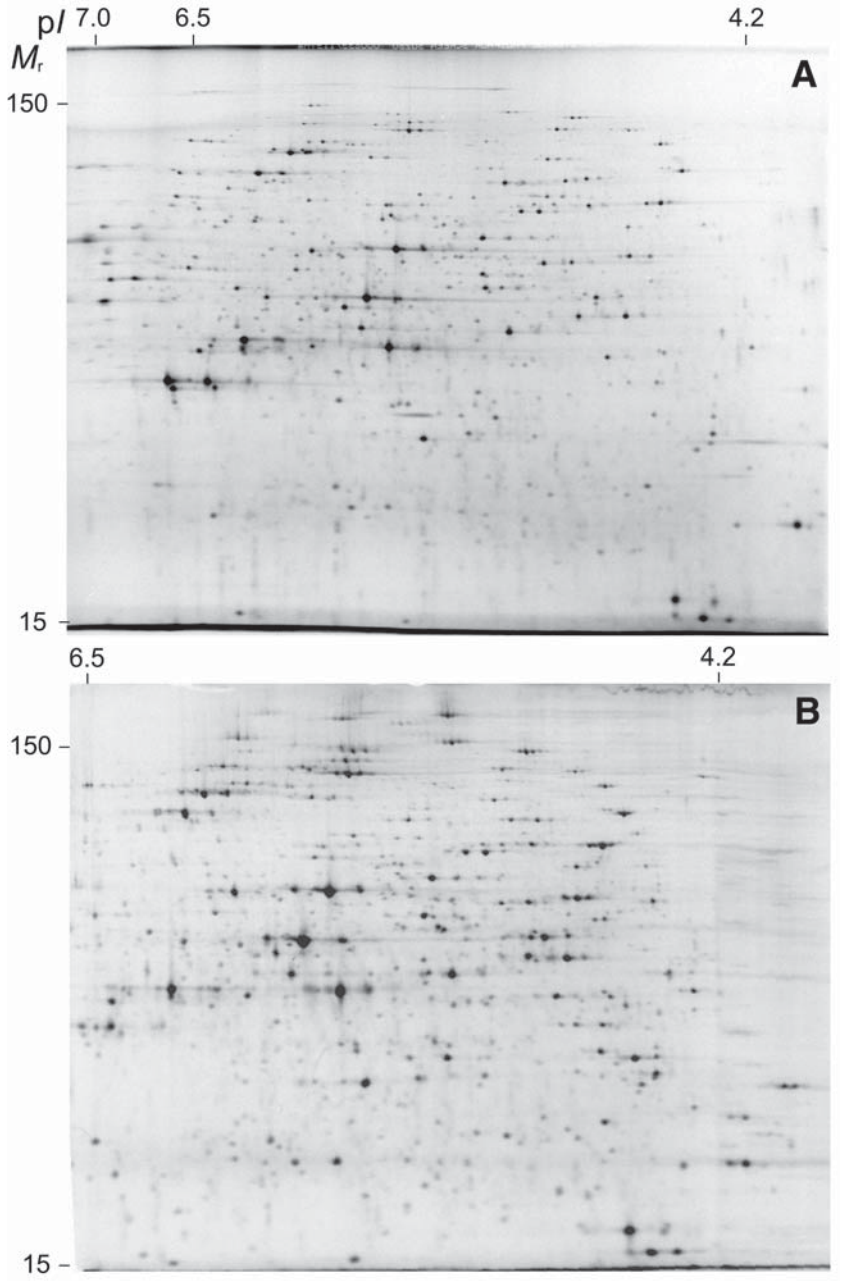

Fig. 2. Two-dimensional gel separation of proteins synthesized in vivo by *Saccharomyces cerevisiae* exponentially growing cells. Proteins were separated in the first dimension on IEF gel **(A)** or on IPG gel **(B)**. Exponentially growing cells were labeled for 10 min with [^{35}S]-methionine. After protein migration, gels were dried and exposed for one night to phosphor screens. Isoelectric points (p*I*) and M_r have been estimated as in **ref.** ***11***.

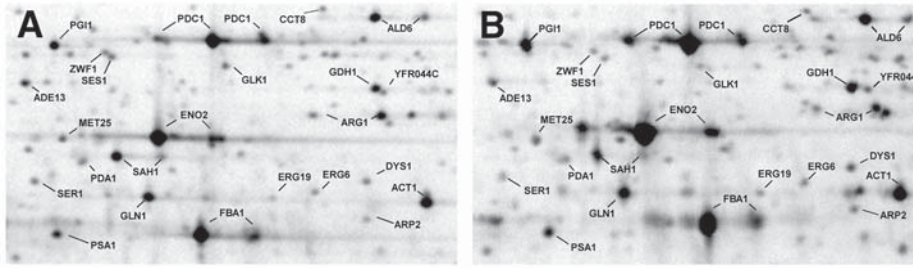

Fig. 3. Identified proteins on a corresponding detail of the protein patterns obtained according to the two 2-DE methods. Proteins were separated in the first dimension on IEF gel **(A)** or on IPG gel **(B)**.

All solutions should be prepared in deionized water that has a resistivity of 18 MΩ-cm, except culture medium, which is prepared with distilled water. Our laboratory is equipped with a Milli-Q Water system (Millipore) for deionized water. The reproducibility and quality of gels are highly dependent on the quality of the reagents. Changing the origin of the reagents may affect protein separation. APS, TEMED, urea, thiourea, CHAPS, acrylamide (powder), and bisacrylamide (powder) are kept in a place maintained at 22°C. Acrylamide and bisacrylamide solutions are kept in brown bottles to protect from light at 4°C. Pharmalytes are stored as 500-µL aliquots at 4°C. APS, TEMED, and DTT are changed 3 mo after opening; urea, thiourea, and CHAPS are changed 6 mo after. IPG strips are kept at –20°C.

All aliquots mentioned in this chapter are single-use aliquots. Aliquots thawed once should not be refrozen.

Wear gloves at all times. Wear a filtering respirator to weigh acrylamide, bisacrylamide, and SDS.

2. We observed that the addition of tyrosine (24 µg/mL) in the culture medium stimulates by a factor of 4–6 the rate of radioactive methionine or radioactive leucine incorporation.
3. Radiolabeled [^{35}S]-methionine is used extensively for labeling yeast proteins because it is commercially available at a high specific radioactivity (>1000 Ci/mmol) and β-emission is easily detectable. Another benefit of [^{35}S]-methionine is that the small intracellular pool of methionine allows reaching isotopic equilibrium very rapidly, in less than 30 s ***(13)***. However, some yeast proteins may be devoid of methionine (removal of the N-terminal methionine is the rule in yeast). When interested in such proteins, radiolabeled [^{14}C]-leucine can be used.
4. Note that the glycine concentration of the cathodic solution for running second-dimension gels with IPG strips is twice the standard concentration. We observed that doubling the glycine concentration improves the migration of high molecular-weight proteins in the second dimension when IPG strips are used for the first dimension.

5. The labeling conditions have been defined such that the incorporation of [^{35}S]-methionine remains linear over the 10-min labeling period (if the culture conditions are different, it is strongly suggested to check whether incorporation is still linear, and to adjust the parameters of the labeling solution if it is not the case). The difference in culture sample volume used for protein labeling depending on the type of first dimension that will be used (IEF or IPGE) is based on the fact that higher amounts of proteins are required to be loaded on IPG gels for obtaining round shape spots on the final 2-D pattern.
6. It is important not to lyophilize cells as a pellet because it would greatly impair the efficiency of cell breakage.
7. Overpassing 4 h of lyophilization decreases cell disruption efficiency.
8. The final volume of the sample is 375 µL and protein concentration is around 1 µg/µL. When applied to exponentially growing cells, the labeling procedure described here leads to the incorporation of about 500,000 cpm/µL of lysate when the lysate is prepared for IEF migration and half as much when the lysate is prepared for IPG migration.
9. TEMED is not required for polymerization.
10. Urea may precipitate in the tube during polymerization. The precipitate will disappear during focusing.
11. The amount of protein or radioactivity to be loaded depends on the experiment. Typical loading for IEF gels with radioactive proteins is 15 µg of protein corresponding to 7.5×10^6 cpm. Up to 200–300 µg of protein can be loaded on IEF gels if one wants to detect unlabeled proteins with Coomassie Blue or silver staining, but increasing the amount will shorten the pH gradient on the basic side.
12. While gels are waiting for extrusion, they must be kept at 4°C in order to prevent protein diffusion. After some time at 4°C, urea crystallization may be observed. This precipitate increases the risk of breaking the gel during extrusion. If urea has precipitated, warm the tube in your hand before extruding the gel. The urea crystals will rapidly disappear.
13. A stacking gel is not necessary. Note also that the resolving gel does not contain SDS. The only SDS in the slab gel during migration comes from the electrophoresis buffer of the upper electrode chamber. We found that the absence of SDS in the slab gel has a "stacking" effect on proteins when they leave the first-dimension gel to enter the slab gel.
14. Equilibration of the first-dimension gel prior to the second dimension is not necessary. Owing to the use of a vertical second dimension system, sealing the first dimension on top of the slab gel with agarose is not required.
15. It is strongly recommended to register the running parameters during focusing by connecting the Focusing Cell to a printer (Bio-Rad). A typical evolution of amperage during focusing is shown in **Fig. 4**.
16. Generally an overnight exposure is enough. For quantitative analysis of the whole proteins separated on a gel, the gel is exposed until the peak intensity of the actin spot reaches half the saturation value of the screen. Under this condition, saturation of the screen is observed for only three spots, corresponding to the major spots of Eno2p, Pdc1p, and Tdh3p.

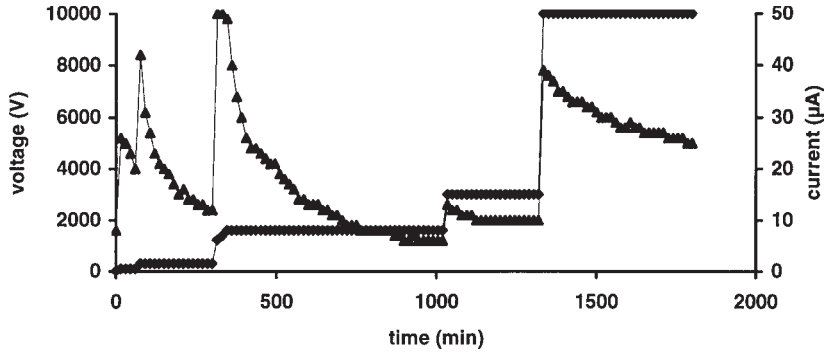

Fig. 4. Time course of voltage and amperage during IPGE. The immobilized pH gradient was from 4.0–7.0 and the IPG strip was 24 cm long. Voltage, (◆); amperage, (▲).

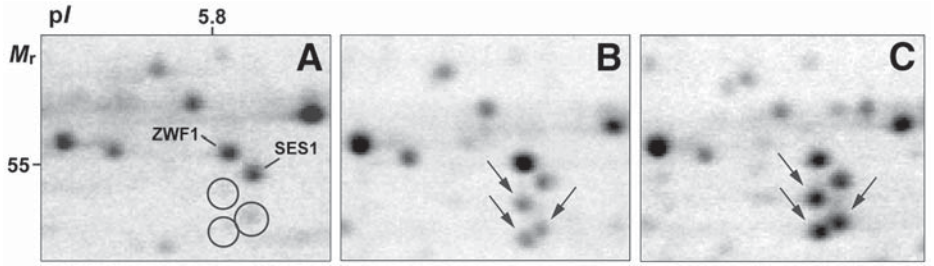

Fig. 5. Spots indicators of proteolysis. The location of the three spots indicative of the degradation status of yeast cellular extract is given by circles in **(A)** and by arrows in **(B)** and **(C)**. In the absence of proteolysis, these three spots are almost undetectable **(A)**. Their abundance is markedly increased when proteolysis occurs (**B** and **C**). Twenty artefactual spots were detected on the 2-D pattern corresponding to **(B)**, and more than 160 on the protein pattern corresponding to **(C)**. The spots Zwf1p and Sse1p, which are close to these spots, are indicated to help the location of the three spots on the whole 2-D pattern (*see* **Fig. 3**).

17. Artefactual spots have three main origins: carbamylation, degradation, and contamination.
    a. Abnormal raw of spots with the same molecular weight: (1) proteins are carbamylated by isocyanate. Use pure urea, freshly prepared urea solution, and avoid high temperatures when proteins are in the presence of urea; (2) some protease inhibitors are known to induce charge alteration.
    b. Proteolysis. It is extremely difficult to completely avoid proteolysis. Strong proteolysis is easily detectable because it results in an abnormal proportion of low molecular weight proteins (the average M_r of yeast proteins is 45,000). A

limited proteolysis remains more difficult to detect. A good indicator is the presence on the yeast protein pattern of three minor spots located close to the ZWF1 and SES1 spots (**Fig. 5**). These spots are degradation products of one of the major yeast proteins, Pdc1p ***(11)***. Their abundance is indicative of the degree of degradation of cellular extract. Normally they are either absent or extremely faint. Their clear detection is always associated with the occurrence of additional degradation products. There are two critical steps where proteolysis can occur: sample preparation, and in-gel rehydration of the sample when running IPG gels.

c. Contamination. Because radioactivity is a very sensitive method for protein detection, it allows the detection of minor contaminants. If "new" spots are mainly concentrated on the acidic side of the gel, there is some possibility that they are corresponding to bacterial proteins. Always check for the presence of contaminants in the culture prior to labeling yeast cells!

Acknowledgments

The authors are grateful to Aurélie Massoni for excellent technical assistance and to Michel Perrot for help in preparing the artwork.

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8

Pulsed-Field Gel Electrophoresis of Budding Yeast Chromosomes

Laura Maringele and David Lydall

Summary

Pulsed-field gel electrophoresis (PFGE) can be used to separate the 16 budding yeast chromosomes on the basis of size. Here we describe a detailed, practical protocol that will allow a novice to perform informative PFGE experiments. We first describe the culture of yeast prior to analysis, along with details of embedding cells in agarose before removal of cell walls. We then detail the procedure to remove protein and RNA from chromosomes and how naked chromosomes are loaded into agarose gels before being subjected to electrophoresis. Finally, we describe how the separated chromosomes can be visualized and photographed.

Key Words: Chromosome size; *Saccharomyces cerevisiae*; restriction enzyme; pulsed-field gel electrophoresis; PFGE; karyotype; budding yeast; CHEF-DR III; Bio-Rad.

1. Introduction

Pulsed-field gel electrophoresis (PFGE) is a reliable method to separate DNA fragments that are too large to resolve by conventional agarose gel electrophoresis, particularly 50 kb and larger ***(1)***. PFGE readily lends itself to the task of resolving the *Saccharomyces cerevisiae* chromosomes, which vary in size between 200 and 1500 kb. Indeed, the first use of PFGE was to resolve the chromosomes of budding yeast ***(2)***. Resolved chromosomes can be observed after staining DNA with dyes such as ethidium bromide or SyBr gold ***(3)*** and/or further processed as Southern blots. For example, Liti and Louis recently used PFGE to examine the electrophoretic karyotypes of strains defective in telomerase and *NEJ1* ***(4)***. Similarly, we have used PFGE electrophoresis to examine the karyotypic changes that occur in budding yeast that are maintaining linear chromosomes in the absence of telomerase and recombination (**Fig. 1**) ***(5)***. Here we describe in detail our experimental approach to PFGE.

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

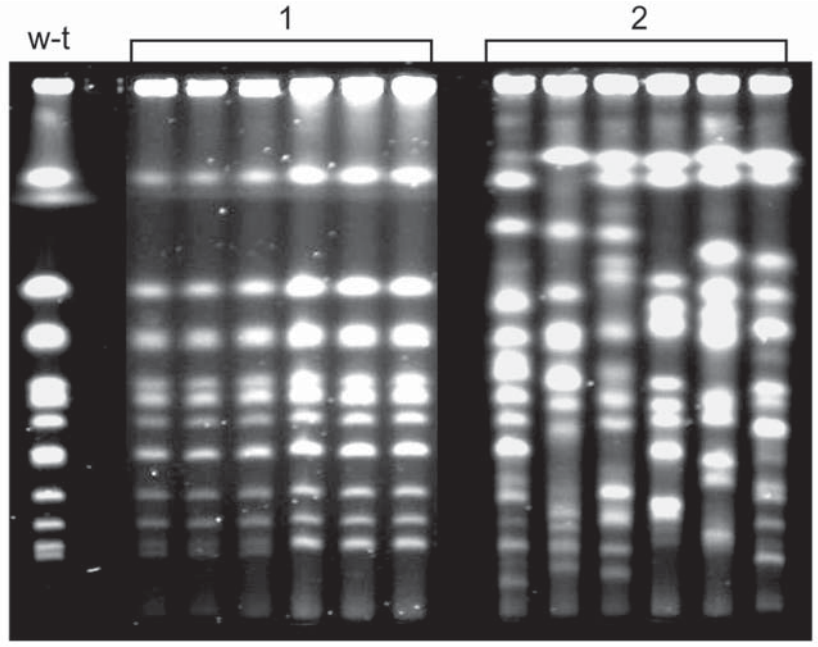

Fig. 1. Pulsed-field gel electrophoresis (PFGE) of whole chromosomes. Cells from a wild-type strain (w-t) and several independent strains defective in telomerase (*tlc1Δ*), recombination (*rad52Δ*), and Exonuclease I (*exo1Δ*) were subject to PFGE and stained with EtBr. Strains shown in lanes bracketed by 1 had been in culture for 4 d after they were taken from a fresh germination plate. Strains shown in lanes bracketed by 2 had been in culture for more than 200 d since germination and each shows a different chromosome size pattern. The three lanes on the right under each bracket also contained an *mre11Δ* mutation.

It is not necessary to have a deep understanding of the principles of PFGE in order to perform useful experiments. In fact, we initiated experiments with only the barest knowledge of the principles underlying this technique. For those who are interested, there are several plausible theoretical explanations for the behavior of large molecules when subject to PFGE ***(1,6)***.

We have used a variation of PFGE electrophoresis called contour-clamped homogeneous electrophoresis (CHEF) ***(5,7)***. The practical advantage of CHEF over other types of PFGE is that the DNA runs in a linear direction, rather than a curvalinear, arc-like, or other trajectory ***(7)***. Although other types of PFGE also separate DNA molecules in straight lines, for most practical purposes yeast geneticists use the CHEF system to resolve and observe budding yeast chromosomes. We have used a Bio-Rad CHEF-DR III machine to perform CHEF. However, with minor modifications, it should be possible to adapt our protocol

to use other commercially available CHEF systems. This chapter aims to enable any yeast geneticist to run productive and informative PFGE and should be read as a companion to the instruction manual provided by the manufacturer.

2. Materials

Unless explicitly stated, all solutions and media are autoclaved for 15 min at 121°C and chemicals and biochemicals are from Sigma Aldrich (Poole, UK).

1. YEPD (ade) liquid: 1% Bacto Yeast Extract, 2% Bacto-Peptone, 2% dextrose, 0.0055% (or 55 mg/L) adenine. For 1 L: add 10 g Bacto yeast extract, 20 g Bacto-Peptone to 950 mL deionized water and autoclave. Before use, add 50 mL of 40% (w/v) dextrose solution and 5.5 mL of 1% (w/v) adenine.
2. YEPD (ade) plates: 1% Bacto Yeast Extract, 2% Bacto Peptone, 2% Bacto Agar, 2% dextrose, 0.0055% (or 55 mg/L) adenine. For 1 L (approx 40 plates): add 10 g Bacto yeast extract, 20 g Bacto Peptone, 20 g Bacto agar to 950 mL deionized water and autoclave. Cool to 60°C, add dextrose and adenine as in **item 1**. Cap bottle and invert gently to mix. In a sterile environment, pour approx 25 mL of the warm liquid into each Petri dish. Leave plates to solidify overnight. After plates have cooled and dried, they can be stored in plastic bags at 4°C for months.
3. 40% (w/v) Dextrose. Weigh 400 g D-Glucose; add deionized water to 1000 mL and autoclave.
4. 1% (w/v) Adenine. Weigh 10 g Adenine hemisulphate (Sigma A9126); add deionized water to 1000 mL and autoclave.
5. Ethylenediaminetetraacetic acid (EDTA): 0.5 *M*, pH 8.0. To make 1 L, add 186.1 g disodium EDTA to 800 mL deionized water. Stir vigorously and adjust pH to 8.0 with NaOH pellets (about 20 g) and autoclave.
6. EDTA: 50 m*M*, pH 8.0, by dilution of 0.5 *M* EDTA stock in autoclaved deionized water.
7. EDTA: 0.125 *M*, pH 7.0. Dilute stock EDTA solution (0.5 *M*, pH 8.0) and adjust pH to 7.0 with concentrated HCl. Autoclave to re-sterilize.
8. SCE solution: 1 *M* sorbitol, 0.1 *M* sodium citrate, and 60 m*M* EDTA. To make 500 mL, add 91.1 g sorbitol, 14.7 g Na citrate, and 11.2 g Disodium EDTA to 450 mL deionized water, adjust pH to 7.0 with NaOH pellets, volume to 500 mL with deionized water, and autoclave to sterilize.
9. Low-melting-point (LMP) agarose for sample preparation, 1% in 125 m*M* EDTA, pH 7.0 (*see* **Subheading 3.2., step 1**.).
10. Zymolyase 20T (20,000 U/g; ICN Chemicals, cat. no. 32092). Store dessicated at 4°C.
11. β-mercaptoethanol (14.4 *M*, store in dark).
12. Zymolyase Solution: 1 mL SCE solution, 9 mg Zymolyase 20T, 50 µL β-mercaptoethanol. This volume is sufficient to make 100 plugs (agarose-embedded chromosomes), e.g., five each from 20 different yeast strains and is made fresh just prior to use.
13. Tris(hydroxymethyl)methylamine (Tris): 1 *M*, pH 8.0. To make 1 L, add 121.1 g Tris base and 950 mL distilled water. Adjust pH with about 40 mL of concentrated HCl and autoclave.

14. EDTA-Tris-β-Mercaptoethanol (ETB) Solution: For 10.5 mL ETB solution: 9 mL 0.5 *M* EDTA, pH 8.0, 1 mL 1 *M* Tris-HCl, pH 8.0, and 0.5 mL β-mercaptoethanol. This volume is sufficient for 20 plugs (4 yeast strains) and is made fresh just prior to use.
15. Proteinase K powder (Sigma, cat. no. P6556).
16. RNase A (Sigma, cat. no. R5303). Dissolve dry powder at 10 mg/mL in 10 m*M* Tris-HCl 15 m*M* NaCl, pH 7.5, and boil (100°C) for 15 min to destroy contaminating DNases. Cool to room temperature and store 1-mL aliquots at –20°C until required.
17. *N*-Lauroylsarcosine sodium salt for molecular biology (Sigma, cat. no. L9150). To make 10% stock, dissolve the *N*-Lauroylsarcosine into 0.5 *M* EDTA, pH 8.0, and store at room temperature. There is no need to autoclave this solution.
18. Proteinase Solution. For 10.1 mL: 9 mL 0.5 *M* EDTA, 1 mL 10% *N*-Lauroylsarcosine, 10 mg proteinase K, 1 mg RNase (100 µL of 10 mg/mL RNase solution). This volume is sufficient for 20 plugs (4 yeast strains) and is made fresh just prior to use.
19. Tris-EDTA (TE) buffer. 1X TE is 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0, made by dilution of 100X concentrate from Sigma with sterile deionized water.
20. Disposable Plug molds (Bio-Rad, Hemel Hempstead, UK).
21. 5-mL disposable syringes.
22. Storage Solution. For 10 mL: 9 mL 0.5 *M* EDTA and 1 mL 1 *M* Tris-HCl, pH 8.0.
23. 0.5 X TBE (by dilution of 10X TBE stock). To make 1 L 10X TBE, add 109 g Tris base, 55.6 g boric acid, and 3.72 g EDTA disodium salt to 1 L deionized water. Autoclave.
24. Certified Megabase Agarose from Bio-Rad to make 1% gels in 0.5X TBE.
25. Ethidium bromide (EtBr) to stain the gel *after* electrophoresis. Dilute 10 mg/mL stock solution (Sigma, cat. no. E1510), add 50 µL to 1 L deionized water, to make 0.5 µg/mL.
26. We routinely work in the W303 yeast genetic background and use strains that are *RAD5*$^+$.

3. Methods

3.1. Cell Culture and Yeast Preparation

The aim is to produce similar numbers of cells for analysis in each lane of the CHEF gel and to ensure that the chromosome preparations are similarly good for each strain. Stationary-phase yeast are more resistant to cell wall digestion by zymolyase, so harvest cells before they have reached stationary phase.

1. For each strain to be examined, streak for single colonies on agar plates and incubate them 2–4 d at the optimal temperature (in our case, usually 23 or 25°C).
2. Use a sterile toothpick to inoculate a 4 mL YEPD culture for each strain, into 15-mL round-bottomed glass tubes. The amount of cells depends on the growth rate; usually a full toothpick tip is sufficient. Single or pooled colonies can be used as inoculums. After inoculation, the cultures should be slightly turbid.

3. Incubate liquid cultures overnight or until they reach about 3×10^7 cells/mL (by eye, but by comparison with haemocytometer readings; *see* **Note 1**). At this timepoint, prepare the LMP agarose for the plugs (pieces of agarose with embedded cells) as described in **Subheading 3.2., step 1**.
4. Remove 1.5 mL from each culture into Eppendorf tubes and spin for 10 s at top speed, room temperature. Pour off supernatant. To make five plugs for each strain, the top of each cell pellet should correspond to the 50 µL mark on 1.5-mL Eppendorf tubes (*see* **Note 2**).
5. Add 1 mL of 50 m*M* EDTA, pH 8.0, to each cell pellet and gently vortex to resuspend the cells. Spin 30 s at top speed and aspirate the supernatant.
6. Add 100 µL of 50 m*M* EDTA, pH 8.0.
7. To each sample of yeast in EDTA, add 50 µL of Zymolyase Solution (**Subheading 2., item 12**). Add agarose immediately, as described below (**Subheading 3.2., step 2**).

3.2. Agarose Plug Preparation

Because large fragments larger than 50 kb are susceptible to shearing by pipetting, yeast cells are immobilized in agarose before their cell wall and proteins are removed *in situ* and chromosomes are separated on pulsed field gels. The cell wall of yeast is broken down using zymolyase, which is a crude enzyme preparation extracted from a submerged culture of *Arthrobacter luteus*. The primary enzyme in the preparation is β-1,3-glucan laminaripentaohydroylase, which breaks down the β-1,3 glucan linkages in the yeast cell wall to release laminaripentose, although other enzymatic activities are also present in the Zymolyase preparation. After the cell walls have been removed by zymolyase treatment, the cells are incubated for a further period in EDTA in order to chelate divalent cations (Mg and Mn) and thereby reduce nuclease activities in yeast cells, which could cleave DNA and hence break chromosomes. A proteinase K/sarcosine/RNase treatment finally breaks down cellular proteins, membranes, and RNA to allow DNA within the plugs to migrate into the gel when a potential is applied.

1. Prepare the 1% LMP agarose for the plugs as follows. Weigh 0.5 g of LMP agarose (we use Sigma Agarose for Pulsed-Field Electrophoresis: Sample Preparation for molecular biology) and add it to 50 mL of 0.125 *M* EDTA, pH 7.0, in a 250-mL glass bottle. Heat for a few seconds in the microwave to melt the agarose, remove the bottle, cap, invert, and then swirl by hand until agarose is completely dissolved. It is neither necessary, nor recommended to boil the LMP agarose. For immediate use, keep the 1% agarose in a water bath at 50°C. For future use, store the 1% LMP agarose in a 50-mL Falcon tube at room temperature and simply reheat the tube for 2–5 s in a microwave, invert several times to mix, and maintain in water bath at 50°C during plug preparation.

2. To a single freshly prepared yeast sample in EDTA and Zymolyase solution (**Subheading 3.1., step 7**), add 250 µL of 1% LMP agarose from the 50°C water bath. Pipet up and down three times or until the mixture has a uniform color.
3. Immediately pipet the whole mixture (about 450 µL) into five wells from the plug former, fill the well to the top but do not overfill; there should be about 90 µL in each well. Repeat this process for one yeast strain at a time. Then place plugs in a freezer (–20°C) for exactly 5 min to set. Alternatively, leave the plugs on ice to set. For plug formers, we use the Bio-Rad Disposable Plug Molds (Part No. 1703706) that are provided as five strips of 10 wells each, with taped bottoms.
4. When the plugs have set, remove the tape from half of a strip (five wells) and extrude the five identical plugs (made from one yeast strain) directly into the barrel of a 5-mL syringe by pushing the top of the plugs with a mini-spatula. Label the syringe, clean the spatula with deionized water, and wipe it dry. Repeat for each set of plugs until all plugs are transferred into 5-mL syringe barrels (*see* **Note 3**).
5. To each syringe with five plugs, add 2.5 mL ETB solution (**Subheading 2., item 14**). Perform this operation inside a fume hood and wear gloves. While adding the ETB, block the nozzle of the syringe with a gloved finger and pour ETB solution until the barrel is half-full. Gently insert the plunger about 0.5 cm into the barrel while inverting the syringe. Be careful not to press the plunger at any time. Repeat the operation for all syringes and seal the nozzles with parafilm. Place the syringes into a New Brunswick TC7 wheel and incubate at 37°C with gentle rotation for a minimum of 4 h to overnight. (We routinely incubate for 4 h.)
6. In a fume hood, remove the parafilm, then the plunger, and let the ETB solution pour away by holding the syringe barrel vertically, with the nozzle down. Rinse the plugs twice with 5 mL of 50 m*M* EDTA. Process one syringe at a time and wear gloves.
7. To each syringe with five plugs, add 2.5 mL proteinase solution, similar to the method described for ETB solution (*see* **Subheading 3.2., step 5**). It is not necessary to seal the nozzles at this stage but place the syringes horizontally on the rotating wheel TC7 at 37°C and rotate for at least 6 h to overnight.
8. Allow the proteinase solution to pour away through the nozzle, rinse the plugs with 5 mL of 50 m*M* EDTA, and incubate in 3–4 mL 1X TE for 1 h on the same wheel.
9. The plugs are now ready to use (*see* **Subheading 3.4.**) or they can be stored for weeks in storage solution at 4°C, inside syringes with parafilm-sealed nozzles. Always remove the plugs from the storage solution and put them in 1X TE for 1–2 h before use.

3.3. Restriction Enzyme Digestion

If plugs were stored at 4°C in storage solution, remove and incubate each plug in 1 mL 1X TE for 30 min, then pour off and add fresh 1X TE for another 30 min. Transfer each plug (H × W × D is 8 × 5 × 1 mm) to a fresh 1.5-mL

plastic tube and add 500 µL appropriate 1X restriction buffer. Incubate the tubes for 30 min on ice. Remove the buffer and perform restriction enzyme digestion as follows: add 160 µL (4X Plug Volume) restriction enzyme mixture, composed of 40 µL appropriate 5X restriction buffer, 117 µL Sigma (deionized) water, and 3 µL restriction enzyme. Make sure the plug is completely covered by the restriction enzyme solution. Close the 1.5-mL tube and incubate overnight at the appropriate temperature. After restriction enzyme digestion, soak each plug for 1 h at room temperature in 1 mL 1X TE to remove any buffer salts from the plug. The plugs are now ready for insertion into the agarose gel wells and electrophoresis, as described within **Subheading 3.4.**

3.4. Agarose Gel Preparation and Electrophoresis

We routinely use a standard 14 × 13 cm gel with a 15-well comb. Longer or wider gels and different combs are also available to fit the CHEF-DR III system, e.g., *see* results obtained by Liti and Louis ***(4)***.

1. Pour the gel. Place the platform into the casting stand (make sure that the black mat that belongs to the electrophoresis cell is inside of the casting stand) and tighten the screws. Place the comb on top of the stand (we usually adjust the length of the comb teeth to 17–18 mm using the hand screw). Boil 100 mL of 1% agarose (*see* **Subheading 2., item 24**) in 0.5X TBE, and cool to 60°C. Keep 1 mL of agarose solution into a 1.5-mL Eppendorf tube at 60°C to seal the wells after the plugs are inserted. Pour the remaining agarose solution into the casting stand and allow to set for 1 h or more.
2. While the gel is setting, pour 2 L of 0.5X TBE into the electrophoresis cell. Switch on the power; set the pump at setting 80 (greater than 0.75 L/min), and the temperature to 14°C. Leave this running for 1 h to equilibrate.
3. Set the chosen running parameters for the control panel (*see* **Subheading 3.4., step 6**).
4. The comb holder is gently removed. Sample plugs are gently inserted into the gel wells by gloved hand, with help from a mini-spatula. If the plugs are slightly above the surface of the gel, cut off the plug tops by sliding a sharp razor blade immediately above the wells and remove the cut-off plug tops from the gel surface. Gently press the gel around the plugs with two fingertips to remove the air bubbles from the wells. Seal the top of the wells, including the empty wells, using the saved 1 mL of 60°C agarose. Remove excess agarose from underneath the black mat with a clean tissue.
5. The gel with inserted sample plugs is now placed with the black mat underneath it into the electrophoresis cell.
6. Start electrophoresis.
   We use standard conditions, as recommended by the Bio-Rad CHEF-DR III manual, to separate whole budding yeast chromosomes (*see* **Note 4**). If the chromosomes have been cut, vary the conditions according to the size of the expected

fragments, as described in the manufacturer's manual.
Temperature: 14°C
Switch time: 60–120 s (ramped over 24 h)
Run Time: 24 h
Angle: 120°
Voltage gradient: 6 V/cm

3.5. Staining and Photography

After electrophoresis is finished, chromosomes can be stained, photographed, and, if necessary, processed as a Southern blot.

1. When run is finished, wear gloves and remove the gel to a sandwich box containing 300 mL of 0.5 µg/mL EtBr solution in deionized water (*Be careful:* EtBr is a mutagen). Incubate 30 min with gentle agitation. Pour off the EtBr solution and de-stain the gel for 1 h with deionized water. Photograph the gel using a trans-illuminator (254–360 nm) and a charge coupled device (CCD) camera (e.g., Alpha Innotech). Save the JPG image to disk so that the contrast and brightness can be adjusted using Photoshop or other similar software. Also print out a hard copy for your notebook.

4. Notes

1. Do not grow strains for longer than 16 h in liquid culture, because the cell wall becomes more resistant to Zymolyase digestion. If your strains have different growth rates, inoculate more cells from strains that grow poorly.
2. Different pellet sizes are adjusted by adding between 100 and 1000 µL from the remaining liquid culture to the cell pellets that are below the 50 µL mark and re-spun for 30 s. This is repeated until all pellets are similarly sized. It is also important to examine your cells under the microscope after the overnight culture, because some budding yeast mutants or cell cycle-arrested cells are larger than wild-type cells. In such cases, estimate the percentage of larger cells and the volume increment and adjust the size of the cell pellet accordingly.
3. It is also possible to use 15-mL plastic tubes and a green, screened cap, sold by Bio-Rad. The number of plugs per 5-mL syringe can be increased to 10, in which case use twice the amount of cells and solutions. If more than 10 plugs are required, use a larger syringe or 15-mL plastic tube and correspondingly increase the volume of cells and solutions.
4. Under the electrophoretic conditions we use, some wild-type chromosomes overlap (*see* **Fig. 1**). Better resolution has been reported using 0.95X TAE buffer (by dilution of 50X TAE from Sigma) instead of 0.5X TBE and using the following conditions ***(8)***.
Temperature: 12°C
Switch time: 60 s for 15 h, 80 s for 7 h
Run Time: 22 h
Angle: 120°

Voltage gradient: 5.9 V/cm

Acknowledgments

We are extremely grateful to colleagues for advice on pulsed field gel experiments. Our initial experiments used equipment and advice from Lesley Lockhart, David Gardner, Daniella Delneri, Andy Hayes, and Steve Oliver at the University of Manchester. Subsequently we received invaluable advice and protocols from Gianni Liti and Ed Louis at the University of Leicester. Our work is supported by the Wellcome Trust.

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9

Analysis of Yeast Lipids

Roger Schneiter and Günther Daum

Summary

The precise quantitative determination of the different lipid classes in mutant cells is key to understand the possible role of the respective gene product in lipid homeostasis. In this chapter, we describe methods based on thin-layer chromatography that are employed routinely to determine the level and relative composition of the major lipid classes from yeast.

Key Words: Lipids; phospholipids; fatty acids; yeast; thin-layer chromatography.

1. Introduction

Changes in relative levels of different lipid classes are the hallmark of various mutants in lipid biosynthesis of *Saccharomyces cerevisiae*. Such global changes in the lipid pattern are easily detectable in whole-cell lipid extracts. However, more detailed analysis of the function of various lipids in different subcellular membranes requires their prior isolation by subcellular fractionation ***(1)***. In this case, the yield of the purified membrane is frequently limiting for lipid analysis. This limitation, however, can be overcome by using sensitive methods for lipid analysis, such as mass spectrometry ***(2,3)***.

A typical lipid extract contains polar and nonpolar lipids. The main components of the polar lipids are glycerophospholipids and sphingolipids. The main components of the nonpolar lipids are free fatty acids, diacylglycerols, triacylglycerols, sterols, and steryl esters.

Different methods are employed for the separation and analysis of different classes of lipids. This section discusses separation of lipids by ascending thin-layer chromatography (TLC) using aluminum foil sheets coated with silica gel adsorbent. Silica gel with a pore size of 60 Å is the most commonly used adsorbent for the TLC analysis of lipids. The technique is simple, versatile, and

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

highly sensitive with the flexibility to be used both quantitatively and qualitatively ***(4)***.

Silica gel is a polar adsorbent. Consequently, polar lipids are more tightly adsorbed than nonpolar lipids. Most nonpolar lipids therefore migrate at the fastest rates (high R_f values), and the polar lipids at the slowest rates. By increasing the polarity of the developing solvent system, the R_f values of components can be increased. No single solvent system will separate all lipid classes. It is therefore important to use different solvent systems to comprehensively analyze the lipid classes or to choose a particular solvent system, depending on the aim of the study ***(5)***.

Because lipids are generally colorless, the separated lipid components have to be rendered visible by chemical reagents. Therefore, the plates must be treated or stained by some method to reveal the position of lipids. The stains used for this purpose can be divided into two main categories: general stains that will enable virtually all lipids to be visualized nonspecifically, and specific stains that will only stain certain types or classes of lipids.

Several methods are used to detect lipids without discriminating between different lipid classes. The most commonly used methods are iodine vapor staining and sulfuric acid charring. Detecting lipid spots by staining with iodine vapor is the most rapid and nondestructive method. Charring is very sensitive and amounts as low as 1 µg of lipid can be detected, but the method is destructive. For quantitative analysis, the proportions of the individual components are determined by various techniques available, such as scanning at 275 nm for quantifying ergosterol or determination of the content of inorganic phosphate present for the quantification of phospholipids.

The methods for selective staining of particular lipids are usually more complicated than the nonspecific stains described earlier. They generally involve a chemical or chemicals in the reagent reacting with specific groups in the lipids that results in the lipid being stained or made visible in some way. Some of the most commonly used reagents and methods to discriminate between different lipids are summarized below.

2. Materials

2.1. TLC Analysis of Lipids

1. Silica gel 60 TLC plates (Merck, Darmstadt, Germany).
2. Microsyringe (Hamilton, Bonaduz, Switzerland) or sample applicator (Linomat IV; Camag, Muttenz, Switzerland).
3. TLC chamber with cover and saturation pads, e.g., Whatman filter paper (Springfield Mill, UK).
4. Solvent systems:

a. For neutral lipid analysis: petroleum ether/diethyl ether/acetic acid (70:30:2; per vol.).
b. For polar lipid analysis: chloroform/methanol/ammonia solution (25%) (50:25:6; per vol.).

5. Incubator set to 100–220°C.
6. Staining reagents:
   a. Nonspecific staining reagents:
      i. For iodine vapor staining: covered TLC chamber containing a few crystals of iodine.
      ii. Sulfuric acid charring: 50% sulfuric acid (v/v) in aerosol spraying device. To be used in a fume hood.
   b. Most commonly used specific staining reagents:
      iii. Molybdenum blue reagent for the detection of phosphate-containing lipids: This stain is available from Sigma (St. Louis, MO) or can be prepared as follows: 40.1 g of MoO_3 is dissolved in 1 L of 25 *N* H_2SO_4 by boiling gently (reagent I). 1.78 g of powdered molybdenum is added to 500 mL of the aformentioned solution and the mixture is boiled gently for 15 min (reagent II). The solution is cooled and decanted. Equal volumes of reagent I and reagent II are mixed and the combined solution is diluted with 2 volumes of water. The final reagent has a greenish-yellow color and is stable for months.
      iv. Dragendorff's test for the detection of choline-containing lipids: This stain is available from Sigma or can be prepared as follows: 40 g potassium iodide is dissolved in 100 mL water (reagent I); 1.7 g bismuth subnitrate is dissolved in 100 mL 20% acetic acid (reagent II); 5 mL of reagent I is mixed with 20 mL reagent II, and then diluted to 75 mL to give the spray reagent.
      v. Ninhydrin spray for the detection of lipids containing free amino groups, i.e., phosphatidylethanolamine and phosphatidylserine. 0.25% ninhydrin in ethanol (available from Sigma).
      vi. $MnCl_2$ charring for the detection of neutral lipids: 0.63 g $MnCl_2 \cdot 4H_2O$, 60 mL water, 60 mL methanol, 4 mL conc. sulfuric acid, in a diving chamber.

2.2. Two-Dimensional TLC Separation and Quantification of Different Glycerophospholipid Classes

1. Silica gel 60 TLC plates (Merck).
2. Sample applicator (Camag) or microsyringe (Hamilton).
3. TLC chamber with cover and saturation pads, e.g., Whatman filter paper (Springfield Mill, UK).
4. Solvent systems:
   a. First dimension: chloroform/methanol/ammonia (65:35:5; per vol.).
   b. Second dimension: chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.).
5. Iodine vapor chamber.
6. Water sprayer.

7. Razor blade or scalpel.
8. Phosphate-free glass tubes. These are either new, unused Pyrex tubes or, if used, need to be boiled in phosphate-free detergent and rinsed with deionized phosphate-free water. They must be heat-stable up to 200°C and should be used for phosphate determination only.
9. Solutions: acid mixture: conc. H_2SO_4/72% $HClO_4$ (9:1; v/v). ANSA solution: dissolve 40.0 g $K_2S_2O_5$, 0.63 g 8-anilino-1-naphthalenesulfonic acid, and 1.25 g Na_2SO_3 in 250 mL water. Ammonium molybdate solution: 0.26% $NH_4(MoO_7)_7 \cdot 4H_2O$.
10. Standard phosphate solutions: 2.68 g of $Na_2HPO_4 \cdot 7H_2O$ dissolved in 1 L H_2O (10 m*M*). This stock solution is diluted 1:10 to give 1 nmol P_i/µL.
11. Heating block in fume hood.
12. Water bath set to 90°C.
13. Spectrophotometer for measurements in the visible light spectrum.

3. Methods

3.1. TLC Analysis of Lipids

1. A fine spotting line is drawn with a pencil 2–3 cm from the bottom of the activated TLC plate (*see* **Note 1**). 10–80 µg of the lipid sample is applied using either a microsyringe or a sample applicator device as spots or lines 3–5 mm in diameter in a solvent that is as nonpolar as possible (*see* **Note 2**).
2. The TLC plate is left at room temperature for 1–2 min to allow the residual solvent to evaporate. Then the plate is placed in the saturated TLC chamber containing the solvent system required and left there for ascending chromatography until the solvent front reaches about 1 cm from the top of the plate (*see* **Notes 3** and **4**).
   Separation of neutral lipids: Nonpolar lipids are separated on TLC plates using the solvent system petroleum ether/diethyl ether/acetic acid (70:30:2; per vol.). This system separates neutral lipids into monoacylglycerols, diacylglycerols, sterols, triacylglycerols, and steryl esters (ascending order; *see* **Fig. 1**). The polar glycerophospholipids remain at the origin.
   Separation of polar lipids: Polar lipids are resolved one-dimensionally on silica gel using the solvent system chloroform/methanol/ammonia solution (25%) (50:25:6; per vol.). This solvent system results in a limited separation of the different phospholipid classes: phosphatidylserine/phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine/cardiolipin (ascending order; *see* **Fig. 2**). The nonpolar lipids and fatty acids migrate at the solvent front.
3. After separation is complete, the plate is allowed to dry in a fume hood for at least 30 min. Then the separated lipid classes are visualized by incubating the plate with different reagents as required and the lipids are identified by comparison to standards (*see* **Note 5**).
   a. Nonspecific staining methods:
      i. Iodine stain: The dry plate is placed into a covered TLC chamber containing crystals of iodine. After incubation for a few minutes, yellow or brown

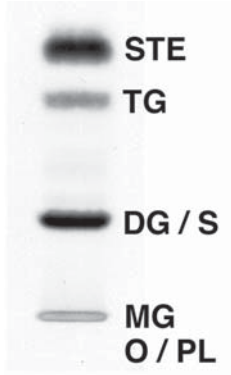

Fig. 1. Example of a TLC separation of neutral lipids. The solvent system was: petroleum ether/diethyl ether/acetic acid (70:30:2; per vol.). Lipids were visualized by $MnCl_2$ charring. PL, phospholipids; MG, monoacylglycerols; DG/S, diacylglycerols/sterols; TG, triacylglycerols; STE, steryl esters; O, origin.

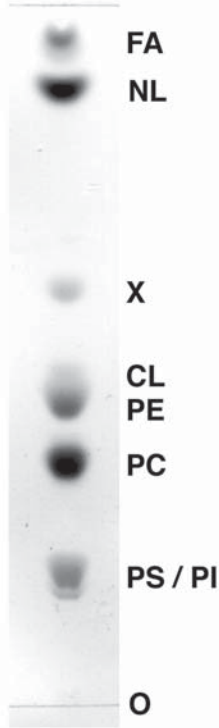

Fig. 2. Example of a one-dimensional TLC separation of polar lipids from *S. cerevisiae*. The solvent system was: chloroform/methanol/ammonia solution (25%) (50:25:6; per vol.). Lipids were visualized by charring. PS/PI, phosphatidylserine/phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; X, unidentified; NL, neutral lipids; FA, fatty acids; O, origin.

spots appear. The plate is removed from the chamber and individual spots are outlined with a pencil before the stain starts to fade (*see* **Note 6**). Most of the naturally occurring lipids are sensitive to this test. However, certain lipids, e.g., completely saturated lipids such as the yeast sphingolipids and some glycolipids of animal origin are not detected by iodine vapor. Stained plates are destained by gently heating with a hair dryer.

ii. Sulfuric acid charring: The dry plate is sprayed lightly with 50% sulfuric acid (v/v) in a fume hood and the plate is then heated at 220°C in an oven for 15 min. All lipid classes will form dark brown or black spots on a white background. This method is very sensitive in detecting lipids and is easy to perform.

b. Specific staining methods:

iii. The phosphate-containing glycerophospholipids can be detected using a molybdenum blue reagent according to a method described by Dittmer and Lester ***(6)***. The plate is sprayed evenly with the reagent. After a few minutes, the phospholipids will appear as dark blue spots on a white or light blue-gray background. Neutral lipids and sphingolipids/glycolipids will not stain.

iv. Choline-containing phospholipids can be detected with Dragendorff's reagent. This test specifically stains phosphatidylcholine and choline-containing sphingomyelin. The TLC plate is sprayed with the reagent. The choline-containing lipid will appear as orange-red spots.

v. Lipids containing free amino groups, e.g., phosphatidylethanolamine and phosphatidylserine can be detected using a ninhydrin spray. The TLC plate is sprayed with a solution of 0.25% ninhydrin in ethanol. After heating the plate at 100°C for 5–15 min, the amino-containing lipids appear as pink-purple spots.

vi. $MnCl_2$ charring for the detection of neutral lipids: The dry plate is dipped in a methanolic $MnCl_2$ solution and then heated in an oven at 100°C for 30 min. Neutral lipids will form dark brown or black spots on a white background. This method is very sensitive in detecting neutral lipids and is easy to perform.

3.2. Two-Dimensional TLC Separation and Quantification of Different Glycerophospholipid Classes

Different glycerophospholipid classes, especially phosphatidylserine and phosphatidylinositol, are not completely resolved on a one-dimensional TLC system. It is therefore necessary to use two-dimensional systems run at right angles to each other to completely separate these lipids for subsequent quantification. On these two-dimensional systems, only one sample can be analyzed per plate. Identification of different lipids is done by comparison to standards run in the same solvent system.

Phospholipids are quantified by determining their phosphorus content. This is achieved by hydrolyzing the phospholipids in anhydrous acids followed by the determination of the content of inorganic phosphate (P_i). Below, a typical

protocol to separate and quantify the glycerophospholipid classes routinely used in our laboratory is described *(7)*.

1. Usually 500 µg of total lipids are applied as a spot of 3–5 mm diameter around 2–3 cm from the left bottom edge of the plate. After evaporation of the solvent, the plate is developed in the first dimension using chloroform/methanol/ammonia (65:35:5; per vol.) as the solvent.
2. When the solvent front is within 1 cm of the top of the plate, the plate is removed from the chamber and allowed to dry completely until the smell of ammonia has disappeared (*see* **Note 7**). This can be accelerated by gently drying the plate with a hair dryer. Be careful not to overheat the plate.
3. The plate is then turned counter-clockwise by 90° so that the lipid spots are now separated in one dimension along the bottom of the plate, and the plate is developed in the second solvent consisting of chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.).
4. After the solvent front has reached within 1 cm of the top of the plate, the plate is removed from the chamber and allowed to dry.
5. Phospholipids are detected by staining in iodine vapor and the spots are marked by a pencil. The identity of the different lipid spots is shown in **Fig. 3**. At this stage, the plates can be stored overnight.
6. The plate is moistened by spraying with distilled water, the phospholipid spots are scraped from the TLC plate using a sharp razor blade or scalpel, and the silica gel is transferred quantitatively to high-quality, phosphate-free glass tubes (e.g., Pyrex). It is not necessary to remove the iodine stain for this purpose. Scrap silica gel from an area of the plate where there is no detectable lipid spot as a blank. The total phospholipid content of the lipid extract is measured directly by taking an aliquot of the extract, evaporating it to dryness, and subjecting it to the acid hydrolysis described below.
7. Add 0.4 mL of acid mixture (conc. H_2SO_4/72% $HClO_4$ [9:1; v/v]) to each tube. Hydrolyze the content by incubating the loosely capped tubes at 180°C in a heating block or sand bath for 30 min. This must be performed in a fume hood, because the hot acid will give rise to very irritating fumes. After hydrolysis is complete, the contents of each tube should turn colorless or light yellow. If some tubes still have dark contents, they may be heated for an extra time or, after cooling, can be bleached by the addition of 10 µL of 30% H_2O_2.
8. Cool the content of the tube and add 9.6 mL of a freshly prepared solution of 500 mL ammonium molybdate (0.26%) and 22 mL ANSA. Vortex and incubate the tube at 90°C for 20 min in a water bath.
9. After cooling and a short centrifugation at 1000*g* to pellet the silica gel, the intensity of the blue color that has developed is measured at 830 nm against the blank.
10. Determine the µmoles of P_i present in the lipid samples by comparison to a standard curve. This standard curve is made by measuring the absorbance of known amounts of P_i from the standard phosphate solution, e.g., 10, 50, and 150 nmoles P_i.

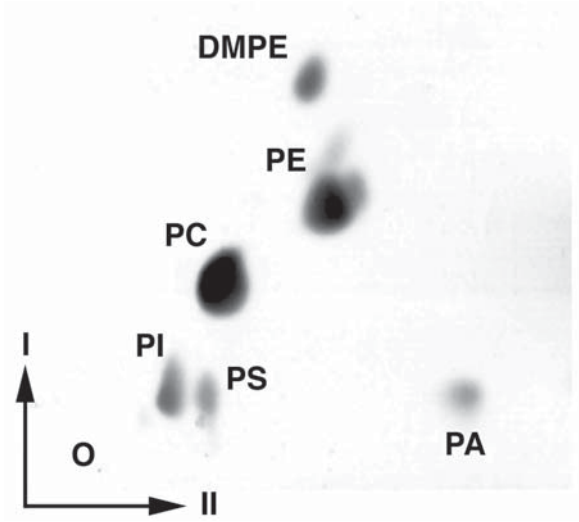

Fig. 3. Example of a two-dimensional TLC separation of the major glycerophospholipid classes. The direction of the first (solvent system: chloroform/methanol/ammonia; 65:35:5; per vol.) and second (solvent system: chloroform/acetone/methanol/acetic acid/water; 50:20:10:10:5; per vol.) dimension is indicated. The lipids were visualized by charring. PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; PC phosphatidylcholine; PE, phosphatidylethanolamine; DMPE, dimethylphosphatidylethanolamine; O, origin.

4. Notes

1. At a given humidity, the amount of water adsorbed by the silica gel increases as pore size decreases. The water content of silica gel determines the polarity of the adsorbent and hence its activity and chromatographic properties. For good separations, the water content of the silica gel therefore must be carefully controlled. To remove the water, the silica gel on TLC plates is "activated" by heating the plates immediately before use for 10 min at temperatures above 100°C.
2. Unless sample solubility requires the use of chloroform/methanol (2:1; v/v), methanol should not be used for sample application, because it tends to produce large spots and wide streaks.
3. Only the purest solvents are used in the development of the plates, and the component solvents should be thoroughly mixed. When solvent systems containing large proportions of polar solvents such as methanol are employed, the chambers can be lined with filter paper to help saturate the atmosphere. However, with nonpolar solvents such as petroleum ether or diethyl ether, the lining of chambers is not necessary.
4. The time taken for a TLC plate to develop depends on the ambient temperature and the solvent system employed. For example, with a solvent system to separate nonpolar lipids, a standard 20×20 cm plate will be developed fully in approx 30

min at room temperature, whereas twice as long is typically required for systems containing polar solvents, e.g., the one required for the second dimension of a two-dimensional separation of glycerophospholipid classes.

5. Although lipid classes can be identified by reference to published R_f values, the application of commercially available lipid standards, either as mixtures or individually, alongside the lipid being analyzed, greatly aids in the identification of the components present in the lipid sample. Within any laboratory, the R_f values of lipid classes in a given solvent system are not always constant owing to day-to-day variations in temperature, humidity, and perhaps even the batch of plates used. By routinely analyzing lipid standards alongside samples, such variations can be taken into account.
6. If the lipids visualized by iodine vapor are to be counted for radioactivity, it is important to let the iodine fade completely before scintillation counting because iodine is an efficient quencher. If the lipid spots are to be assayed by a colorimetric method, then the presence of residual traces of iodine will not pose a problem.
7. For the two-dimensional separation of glycerophospholipids, it is important that all traces of the solvents used for development in the first dimension are evaporated from the plate before it is developed in the solvent system of the second dimension. Although this can be achieved by use of an unheated stream of air from a hair dryer, vacuum desiccation is less likely to damage the lipids on the plate. With all solvent systems used for two-dimensional separation of polar lipids, neutral lipids are observed as a single zone near the top corner of the developed plate when total lipid is analyzed (*see* **Fig. 3**).

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10

Yeast Fluorescence Microscopy

Jiří Hašek

Summary

Fluorescence microscopy is the essential technique for investigation of the intracellular distribution of macromolecules and various organelles also in yeast cells. In this chapter, detailed practical procedures for fluorescence microscopic observations developed or adopted in our laboratory are described. These include labeling of the cell wall and chitin, F-actin structures, nuclear and mitochondrial DNA, and two different procedures for investigation of yeast cells by immunofluorescence. In addition, our experience with multicolor labeling experiments is introduced and discussed.

Key Words: Yeast; fluorescence microscopy; immunofluorescence; multicolor labeling; chitin; actin; microtubules; cell wall; nucleus.

1. Introduction

Fluorescence microscopy is an important technique for visualization of cellular organelles and localization of macromolecules. In yeast cells, such microscopic analysis is a particular challenge owing to a small cell size and the barrier of the cell wall. Basic methods for yeast cells are now well-established ***(1–3)*** and recent developments of various procedures of intragenous tagging of particular gene products have been a great advance (*see* **refs. *4–7***). A routine use of sensitive digital cameras on epifluorescence microscopes or the confocal laser scanning microscopes in the analysis of yeast cells has significantly simplified capturing of images and improved the quality of gained information.

In living yeast cells, a number of various fluorescent dyes can be used for specific labeling of cellular organelles. Besides DAPI (Sigma-Aldrich) to visualize nuclei, these include FM4-64 (Molecular Probes) to show vacuoles ***(8)***, $DIOC_6$ (Molecular Probes) to label endoplasmic reticulum (ER) and mitochondria ***(9)***, DASPMI (Molecular Probes) to reveal mitochondria ***(10)***, and the brightener Calcofluor White (American Cyanamid Co.) to stain the cell

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

wall ***(11)***. A complete review on dyes suitable for yeast cells has been published elsewhere ***(4)*** and is beyond the scope of this chapter.

To localize various macromolecules in fixed cells, indirect immunofluorescence microscopy is the most suitable procedure. In the first step, a primary antibody specifically binds to intracellular epitopes. In the second step, the epitope-bound primary antibody is recognized and visualized by a secondary, fluorochrome-labeled antibody (conjugate). The immunodetection has been simplified by development of various techniques of intragenous tagging of proteins under study with heterologous tags, e.g., fragments of influenza virus hemagglutinin (HA-tag) and the c-Myc proto-oncogen (myc-tag) ***(6)***. Because the antibodies against tags are commercially available, it is not necessary to produce other specific primary antibodies.

For conjugate preparation, fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate were the most widely used discrete labels. Tagged fluorescein gives a strong green-yellow fluorescence. Rhodamine emits a bright red-orange fluorescence. Because these dyes should be stabilized against fading, more photostable fluorochromes have been developed and linked to secondary antibodies. We have had good experience with Cy3 conjugates. In addition, there are conjugates with other fluorochromes (e.g., Bodipi, Texas red, various Alexa Fluor dyes, Cy5) available on the market.

Phalloidin (M_w 800) is a toxin of the mushroom *Amanita phalloides*. Because of its high affinity for polymerized actin (F-actin) ***(12)***, phalloidin tagged with the fluorescent dye is a unique compound that is generally used to monitor distribution of actin microfilaments in various eukaryotic cells. Because this drug does not penetrate into all living cells, the cells should be usually fixed and permeabilized before labeling.

In this chapter are provided detailed protocols for an indirect immunofluorescence microscopy, labeling of F-actin with the rhodamine-tagged phalloidin, and staining of chitin with the FITC-tagged wheat-germ agglutinin that we have used in a variety of different specific applications. In addition, introduced here are some interesting examples of a combination of these procedures with the microscopic analysis of proteins intragenously tagged with the green fluorescent protein (GFP) in multicolor experiments that we have performed in my laboratory. We have been routinely using a BX-60 Olympus fluorescence microscope (Olympus, Tokyo, Japan) equipped with specific filter sets: U-MNUA (excitation 360–370 nm; emission 420–460 nm), U-MWIBA/GFP (-excitation 460–490 nm; emission 510–550 nm), U-MWIY (excitation 545–580 nm, emission 600 nm). A 100×/1.4 n.a. oil-immersion objective was used. The images were recorded with 1280 × 1024 pixel resolution using the Fluoview™ cooled digital monochrome camera and the analySIS™ software.

2. Materials

2.1. Staining of DNA

1. DAPI (4', 6-diamidino-2-phenylindole) (excitation 360–370 nm, emission 420–460 nm): store as 1 mg/mL stock solution of DAPI in H_2O at –20°C until needed; Prepare working solution of 10 µg/mL DAPI in H_2O. Store at 4°C.
2. Tris-based mounting medium: prepare 0.1 % (w/v) *p*-phenylenediamine (Sigma) in 100 m*M* Tris-HCl, 100 m*M* NaCl, 5 m*M* $MgCl_2$, pH 9.5; and 0.4 µg/mL of DAPI (add 40 µL of the DAPI working solution into 1 mL of the buffer). The solution deteriorates. Prepare before use.

2.2. Staining of Actin With Rhodamine-Tagged Phalloidin

1. Rh-phalloidin: stock solution of rhodamine-tagged phalloidin in methanol (R-415; 6.6 µ*M*; Molecular Probes, Eugene, OR). Store in aliquots at –20°C.
2. Tris-based mounting medium: *see* **Subheading 2.1., item 2**.

2.3. Fluorochroming the Cell Wall

1. Calcofluor white (excitation 360–370 nm, emission 420–460 nm): prepare 1 mg/mL stock solution of Calcofluor white (American Cyanamid, Parsippany, NJ) in H_2O and keep in dark at 4°C for months. Filter before use; working concentrations range from 0.01 to 1 µg/mL.

2.4. Staining of Chitin

1. WGA–FITC: stock solution (125 µg/mL) of the FITC-labeled wheat germ agglutinin that specifically binds to chitin (***16***; L-4895 Sigma-Aldrich, St. Louis, MO). Store at 4°C.
2. Tris-based mounting medium: *see* **Subheading 2.1., item 2**.

2.5. Indirect Immunofluorescence

1. PEM buffer: prepare PEM buffer as a fourfold concentrated stock solution –0.4 *M* PIPES, 20 m*M* EGTA, 20 m*M* $MgCl_2$; pH 6.9 (KOH). Store at 4°C up to 1 mo.
2. 7.4% (w/v) formaldehyde: dissolve 2.96 g of paraformaldehyde in 10 mL of H_2O at 60°C in the presence of 10 µL of 1 *N* KOH; mix the solution with 20 mL of PEM buffer stock solution and adjust the volume to 40 mL. Store at 4°C up to 1 mo.
3. KCP buffer: Prepare 0.1 *M* potassium phosphate-citrate buffer, pH 5.9. Dissolve 11.4 g $K_2HPO_4 \cdot H_2O$ and 3.5 g citric acid $\cdot H_2O$ in distilled water. Adjust the volume to 500 mL, pH 5.9.
4. Bovine serum albumin (BSA): prepare 2% (w/v) BSA in PEM buffer, membrane-filter, and store in aliquots at –20°C.
5. Triton X-100: prepare 10% (v/v) stock solution. Prepare working solutions 0.1–1% (v/v) Triton X-100 in PEM buffer immediately before use.
6. Zymolyase stock: dissolve 10 mg/mL of Zymolyase-20T (Seikagaku Corp., Tokyo, Japan) in H_2O. Store in aliquots at –20°C.

7. Pepstatin stock: prepare the stock solution of 1 mg/mL Pepstatin A (Sigma-Aldrich, St. Louis, MO) in methanol. Store at –20°C. Add to cell suspension in ratio 1:100 (v/v).
8. PK 1/1: mouse monoclonal antibody (MAb) against recombinant Rpg1p/Tif32p/eIF3a ***(13,14)*** was applied in the form of the ascitic fluid clarified by centrifugation just before use. Final dilution is 1:100 (v/v).
9. YOL 1/34 rat MAb against α-tubulin (Serotec, Oxford, UK). Store in aliquots at –20°C. Final dilution is 1:10 (v/v).
10. DM1A: mouse MAb against α-tubulin (Sigma-Aldrich, St. Louis, MO). Store the stock solution in aliquots at –20°C. Final dilution is 1:100 (v/v).
11. GAM/Cy3: goat anti-mouse IgG antibody conjugated with Cy3 (Jackson ImmunoResearch Laboratories, West Grove, IL). Store the stock solution in aliquots at –20°C. Dilute the stock solution 1:200 (v/v) with 1 % (w/v) BSA in PEM.
12. GAR/FITC: goat anti-rabbit IgG antibody conjugated with FITC (Jackson ImmunoResearch Laboratories, West Grove, USA). Store the stock solution in aliquots at –20°C. Dilute the stock solution 1:200 (v/v) with 1% (w/v) BSA in PEM.
13. Phosphate-buffered saline (PBS) 10X: dissolve 80 g NaCl, 2 g KCl, 11.4 g $Na_2HPO_4 \cdot H_2O$, 2 g KH_2PO_4 in 1 L of deinonized H_2O, pH 7.4 (NaOH).
14. The rabbit polyclonal antibody (PAb) against Hcr1p ***(15)*** was applied in a final dilution of 1:200 (v/v).
15. PEI: 0.05 % (w/v) polyethylenimin (Sigma-Aldrich, St. Louis, MO).
16. Tris-based mounting medium: *see* **Subheading 2.1., item 2**.

3. Methods

3.1. Visualization of DNA With DAPI in Fixed Cells

1. Mix the yeast suspension with 3 volumes of ethanol or resuspend pelleted cells in 70% (v/v) ethanol for 30 min.
2. Wash cells with KCP buffer by centrifugation.
3. Mount the cells in the Tris-based mounting medium containing 0.4 µg DAPI/mL.
4. Examine in epifluorescence using the filter set for DAPI: excitation 360–370 nm; emission 420–460 nm.
   Chromosomes display a strong blue-white fluorescence; spots of weaker fluorescence localized in the cytoplasm are mitochondrial nucleoids. DAPI is often used for a complementary labeling of DNA in cells analyzed by immunofluorescence microscopy.

3.2. Labeling of F-Actin

1. Add 100 µL of 37% (v/v) formaldehyde (e.g., Acros Organics, Geel, Belgium) to 900 µL of the cell suspension in an Eppendorf tube (final concentration of 3.7% [v/v] formaldehyde).

2. Fix the cells at room temperature with gentle agitation for 5 min.
3. Wash cells with 1 mL of PEM buffer by centrifugation.
4. Resuspend approx 10^7 cells in 20 µL of 25% (v/v) methanol in PEM buffer.
5. Add 2 µL of the stock solution of rhodamine-tagged phalloidin.
6. Stain in dark for 5–15 min.
7. Wash with PEM buffer.
8. Mount the cells in the Tris-based mounting medium. Medium may contain 0.4 µg DAPI/mL to visualize DNA.
9. Examine the specimen in epifluorescence using the filter sets for DAPI (excitation 360–370 nm; emission 420–460 nm) and rhodamine/Cy3 (excitation 545–580 nm, emission 600 nm).

3.3. Fluorochroming the Cell Wall

1. Spin down the cells.
2. Mix cells with a drop of Calcofluor White in a concentration ranging from 0.1–0.5 µg/mL (*see* **Note 1**).
3. Apply a coverslip and observe under appropriate filter system (Utraviolet; UV).

3.4. Staining of Chitin

1. Take an aliquot of 20 µL of the cell suspension (10^8 cells/mL; either living or formaldehyde-fixed cells) in PEM buffer.
2. Add 5 µL of the WGA-FITC stock solution.
3. Incubate at room temperature for 5 min.
4. Wash cells once with PEM buffer.
5. Mount the cells in the Tris-based mounting medium. Medium may contain DAPI.
6. Examine in epifluorescence using the filter sets for FITC/GFP (excitation 460–490 nm; emission 510–550 nm) and possibly DAPI (excitation 360–370 nm; emission 420–460 nm).

3.5. Indirect Immunofluorescence

3.5.1. Fixation

The fixation protocol detailed here provides reproducible staining of the microtubular system (e.g., with antibody DM1A) and the translation initiation factor eIF3 subunit eIF3a/Rpg1p/Tif32p using the MAb PK1/1 ***(13,14)***.

1. Add one volume of 7.4% (w/v) formaldehyde (PARA) in the twice-concentrated PEM buffer to the cell culture (grown to a density of approx 10^7 cells/mL) in a culture medium.
2. Add the Pepstatin stock 1:100 (v/v).
3. Fix for 120 min with shaking on a reciprocal shaker (*see* **Note 2**).
4. Collect the fixed material by centrifugation (e.g., 500*g*, 4 min)
5. Wash three times with PEM buffer.

3.5.2. Permeabilization

The procedure generally used in our laboratory to permeabilize yeast cells is as follows:

1. Wash fixed cells in Eppendorf tubes with KCP buffer.
2. Resuspend the pellet in 1 mL of KCP buffer supplemented with 20 µL of the Zymolyase stock and 40 µL of the Pepstatin stock.
3. Incubate cells at room temperature for 15–30 min. Check digestion of the cell wall with a phase contrast microscope.
4. Centrifuge wall-less cells free from the digestive mixture and carefully wash twice with PEM buffer.
5. Permeabilize cells with 20 µL of 1% (v/v) Triton X-100 in PEM buffer for 30 s (*see* **Note 3**).
6. Wash once with 1 mL of PEM buffer by centrifugation at 500*g*, 4 min.

3.5.3. Incubation With Antibodies

3.5.3.1. Cells in a Suspension

We prefer to work with cells in suspension, because the material can be better washed to lower the background fluorescence. An almost unlimited amount of labeled cells is available for the microscopic investigation. The following protocol works well using various PAbs as well as MAbs.

1. Resuspend the prepared cells (*see* **Subheading 3.4., step 2**) in 2% (w/v) BSA in PEM buffer (*see* **Note 4**).
2. Take one volume of the cell suspension (typically 50 µL) and incubate at room temperature for 20 min.
3. Add an equal volume of the PEM buffer-diluted primary antibody (e.g., DM1A or PK1/1 diluted with PEM buffer; final dilution as indicated in **Subheading 2.5.**). Generally, dilution depends on the quality of the antibody (*see* **Note 5**).
4. Incubate cells at room temperature for 60 min.
5. Wash the cells three times with 1 mL of PEM buffer by centrifugation (*see* **Note 6**).
6. Apply a secondary antibody diluted in 1% (w/v) BSA in PEM buffer. For preparation of stocks and storage, follow recommendations described in company notes. We can recommend GAM/Cy3 conjugate from Jackson Laboratories. Add 50 µL of the 200-times diluted antibody.
7. Incubate at room temperature for 60 min.
8. After removal of the antibody by centrifugation, wash the cells twice with PEM buffer.
9. Mix the cells with an equal volume of the Tris-based mounting medium (*see* **Subheading 2.1., item 2**), cover with a coverslip, and drain excess medium (*see* **Note 7**).
10. Examine the specimen in epifluorescence using filter sets.

3.5.3.2. Cells Attached to Multiwell Slides

This approach is especially suitable if either a tiny amount of the specific antibody is available or if immunolabeling requires the pretreatment of epitopes with organic solvents. Because of the properties of the surface of the wells, we prefer to use multiwell slides from ICN. We use the following protocol to attach and label the cells:

1. Add 3 µL of 0.05% (w/v) polyethylenimin (PEI) onto each well.
2. Dry at room temperature.
3. Apply a drop of distilled water onto each well, drain with vacuum pump, and air dry.
4. Apply 5 µL of cell suspension onto each well and leave the cells to attach for 5 min. Add a drop of PBS buffer and drain the buffer with vacuum pump. Do not dry!
5. Put the multiwell slide with attached cells into cold methanol (–20°C) for 10 min and when necessary, transfer slide into cold acetone (–20°C) for 30 s.
6. Dry the specimen.
7. Apply a drop of PBS buffer to each well and incubate in a moist chamber for 5 min.
8. Drain an excess of the buffer with vacuum pump (do not dry!).
9. Apply diluted antibody and incubate the specimen in a moist chamber for 60 min.
10. Wash the cells in each well three times with 1 mL of PBS buffer (drain the buffer but do not dry!).
11. Apply the secondary antibody (approx 5 µL per each well) and incubate the slides in a moist chamber in the dark at the room temperature for additional 60 min.
12. Wash the cells in each well with 2 mL of PBS buffer.
13. Add the mounting solution (*see* **Note 7**), drain excess buffer, and seal coverslips on the specimen with nail polish.

3.6. Multiple Labeling

The following experiments are advisable to obtain complementary images of distributions of various cellular components simultaneously in the same cell. Usually, two primary antibodies with a different specificity and developed in different animal species are used. The antigens of interest are rendered visible by appropriate secondary antibodies tagged with fluorochromes having different spectral properties (usually FITC and Cy3) (**Fig. 1**).

1. Apply primary and secondary antibodies according to the protocol detailed above (e.g., PK 1/1 mouse MAb followed by Cy3-conjugated goat anti-mouse IgG antibody) to reveal distribution of the first antigen.
2. Apply the second primary antibody (e.g., rabbit PAb against Hcr1p) to the washed and pelleted cells and incubate for 60 min at room temperature (*see* **Note 5**).
3. Wash the cells carefully with PEM buffer; apply the conjugate (in this case FITC-labeled goat anti-rabbit antibody) and incubate at room temperature for 60 min.
4. After washing, mount the cells in the mounting medium and examine by epifluorescence using appropriate filter sets.

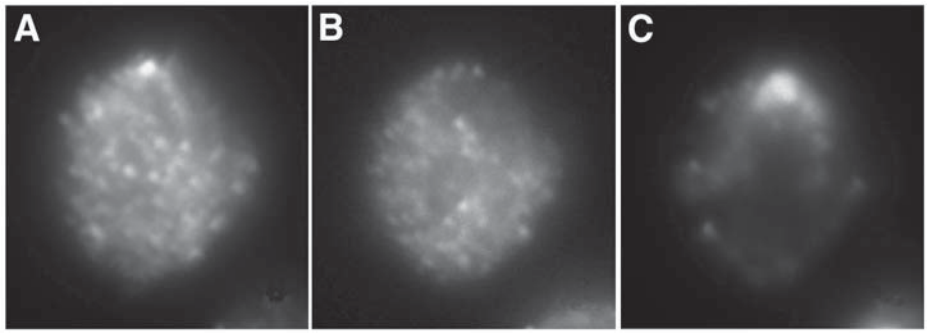

Fig. 1. A multiple-labeled cell of *S. cerevisiae* (strain W303). **(A)** Labeling for Rpg1p with the MAb PK 1/1, **(B)** distribution of Hcr1p revealed by the rabbit polyclonal anti-Hcr1p antibody, and **(C)** staining of DNA with DAPI.

3.7. Co-Localization With F-Actin

For some purposes, the rhodamine-tagged phalloidin can be used in conjunction either with immunofluorescence or in a combination with labeling of other specific compounds or intragenously labeled GFP-fusion. The washed cells after immunolabeling or fixed cells containing GFP-fusion could be co-stained according to the protocol mentioned in **Subheading 3.5.** In this respect, complementary labeling of cells using rhodamine-tagged phalloidin, wheat-germ agglutinin tagged with FITC, and DAPI is one of the best examples of the use of multicolor labeling in fluorescence microscopy of yeast cells (**Fig. 2**).

To visualize F-actin, chitin, and DNA in the same cells, use the following protocol:

1. Fix cells with 3.7% (v/v) formaldehyde for 5 min (add directly the 37% [v/v] formaldehyde stock solution to the cell culture).
2. Wash cells with PEM buffer.
3. Mix 20 µL of the cell suspension in PEM buffer with 2 µL of the Rh-phalloidin stock and 2 µL of WGA-FITC stock.
4. Incubate at room temperature for at least 5 min.
5. Wash once with PEM buffer.
6. Mount cells into the Tris-based mounting medium containing DAPI (*see* **Subheading 2.1., item 2**).
7. Examine by epifluorescence microscope using an appropriate filter set.

3.8. Immunolabeling of Cells Expressing a GFP Fusion

A combination of the immunofluorescence with the microscopic analysis of GFP-tagged proteins in fixed cells is recommended, e.g., for the examination of a possible protein mislocalization owing to the position and the size of the

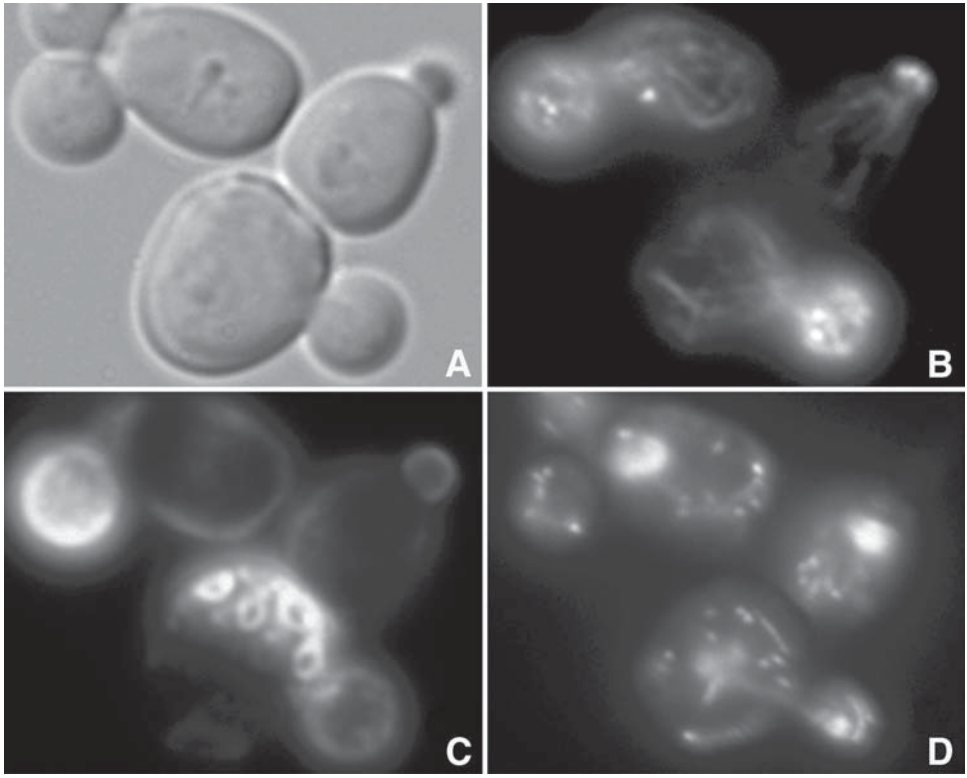

Fig. 2. Multiple-labeled cells of *S. cerevisiae* (strain W303). **(A)** Nomarski image, **(B)** labeling for F-actin with Rh-phalloidin, **(C)** labeling for chitin with WGA-FITC, **(D)** staining of DNA with DAPI.

tag. We performed immunofluorescence microscopy procedure with the specific antibodies on the cells expressing the ER marker Elo3 fused to GFP ***(17)***. The procedure follows the steps mentioned in **Subheading 3.5.3.1.** The appropriate sets of filters should be used for examination (**Fig. 3**). For problems with permeabilization, *see* **Note 3**. Please note that not all GFP fusions can be easily detected after formaldehyde fixation.

4. Notes

1. Application of Calcofluor White includes determination of cell viability, characterization of the porous structure of the cell wall, quantification of bud scars and identification of the localized cell wall growth.
2. The time of fixation can vary depending on the epitopes to be detected. The minimum 45 min time-course of fixation is required to permeabilize cells without alterations detectable by light microscopy.

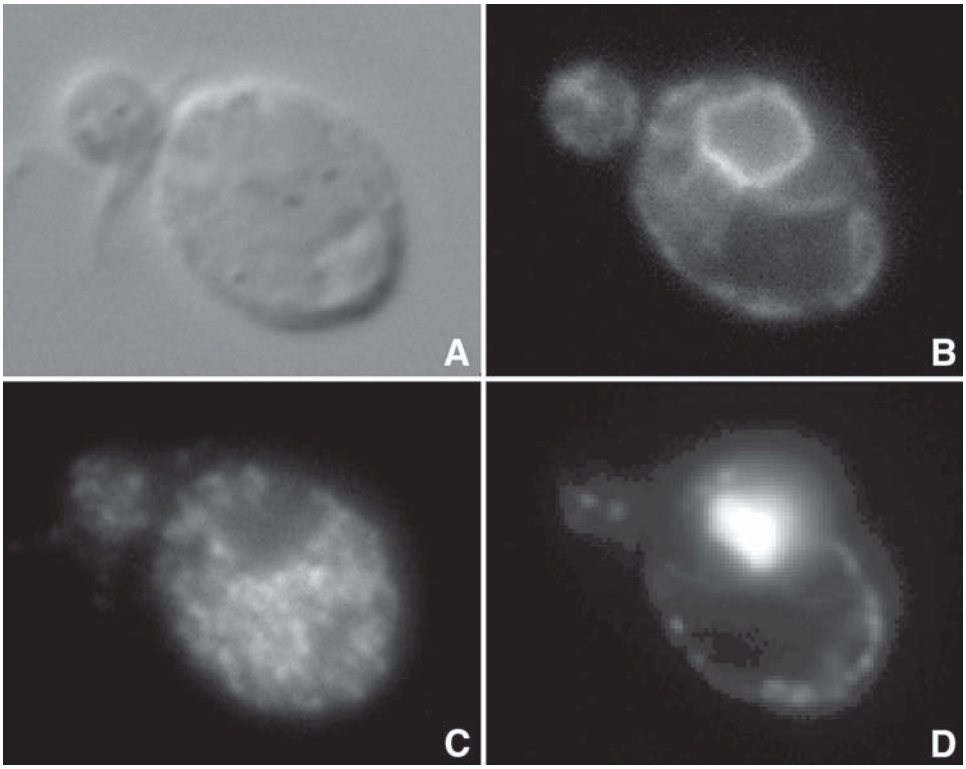

Fig. 3. Immunofluorescence microscopy of the yeast cell expressing Elo3-GFP. **(A)** Nomarski image, **(B)** distribution of Elo3-GFP, **(C)** distribution of Hcr1p, **(D)** staining of DNA with DAPI.

3. We usually use 1% (v/v) Triton X-100 for 30–60 s to permeabilize fixed cells with partially removed cell walls. If there is a possibility that the cytoplasmic antigen could be washed out from the structures or that the labeled structures could be altered during permeabilization with 1% Triton X-100, we recommend the use of a lower concentration (from 0.05–0.1% [v/v] Triton X-100 in PEM). After treatment with this concentration of Triton X-100, distribution of the ER marker Elo3-GFP in fixed and permeabilized cells appeared identical to its distribution in living intact cells. Triton X-100 at higher concentration causes diffusion of the fluorescent signal. Nevertheless, low concentrations of Triton X-100 may not be sufficient to permeabilize the yeast cells for the antibody penetration.
4. We use BSA to diminish nonspecific binding of the antibodies.
5. It is indispensable to have various controls concerning the incubation with antibodies. We recommend the following: (1) examine the reaction of the preimmune serum (if available); (2) in case of intragenously tagged proteins, check the control cells that do not contain the tag; (3) check the cells of the particular deletion

strain (if available); (4) check the cells after a direct labeling with the conjugates alone; (5) check the reaction of the primary antibody pre-absorbed with the antigen (if available); (6) in double-labeling experiments, check the signal of each labeling separately in the other filter system of the microscope.

6. To prevent loss of cells during washing steps, we recommend swing-out rotors for centrifugation.
7. To diminish fading of fluorescein and/or rhodamine fluorescence in response to the effect of the excitation light, *p*-phenylenediamine is used in the mounting medium.

Acknowledgments

I would like to acknowledge Dr. S.D. Kohlwein for helpful discussions. Our work was partially financed by the grants IAA5020102, CSF204/02/1425, and IRCAV0Z50200510.

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11

Intracellular Expression of Recombinant Antibody Fluorescent Protein Fusions for Localization of Target Antigens in *Schizosaccharomyces pombe*

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Summary

Intracellular localization is important for the characterization of a gene product. Microscopy of fluorescent protein fusions has become the method of choice to define the spatial and temporal behavior of a protein. We show here that recombinant antibody fluorescent protein fusions can be used to monitor the localization of intracellular antigens in fixed or living cells. A most successful application of phage-display technology has been the isolation of recombinant antibodies from large combinatorial repertoires. The most versatile antibody format is the single-chain Fv fragment (scFv) in which a flexible polypeptide linker joins the heavy- and light-chain antibody variable domains. Commercial systems are now available to produce scFv phage-display libraries encoding a large pool of binding specificities from which antibodies can be isolated and used as immunochemical or intracellular reagents. We designed a plasmid for ectopic expression of a recombinant antibody fused to a green fluorescent protein (GFP) under the control of an attenuated *nmt1* promoter in *Schizosaccharomyces pombe*. The antibody binds to its target antigen without inhibiting protein function, allowing visualization of its intracellular location in fixed or living cells.

Key Words: Recombinant antibodies; M13 phage display; enhanced green fluorescent protein; thiamine-repressible expression; fluorescent microscopy; cytokinesis; Cdc4p; actin-myosin contractile ring.

1. Introduction

The genome of *Schizosaccharomyces pombe* has 4940 predicted protein-encoding genes ***(1)***. About 31% of these correspond to "unknown" genes, of which half are without homology to any known genes and the other half are conserved genes with unknown function. Intracellular localization is important

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

for the characterization of a gene product. Evidence of changes in intracellular localization, such as translocation to or from the nucleus or time/location dependent accumulation of a protein to form structural complexes such as spindle pole bodies, mitotic spindle, or contractile ring, provides fundamental insights into the function of a protein. Traditionally, methods for protein localization in yeasts include cell fractionation studies, as well as immuno-electron or indirect immunofluorescence microscopy. Recently, microscopy of fluorescent protein fusions has become the method of choice to define the spatial and temporal behavior of a protein in yeasts ***(2,3)***. Ideally, a tagged gene is integrated at its chromosomal locus to place expression of the fluorescent fusion protein under the control of the native promoter. Alternatively, fluorescent protein fusions are expressed from an episome. With the latter approach, timing of expression and concentration may differ significantly from the normal conditions. In both cases, interference from the fluorescent protein with normal protein function or localization is a concern.

We describe a method in which a recombinant antibody fused to a green fluorescent protein (GFP) is expressed within yeast cells. The antibody binds to its target antigen without inhibiting protein function, allowing the tracking of its intracellular location in fixed or living cells. Expression of the antibody-GFP fusion is from an episome (pTRAY; **Fig. 1**) specifically designed to accept single-chain recombinant antibody (scFv) sequences from scFv libraries constructed with commercial M13 phage display kits. There are several advantages to this approach. First, the protein of interest is unmodified and its expression remains under control of the native promoter. Second, there is high specificity of the antibodies for the antigen, because scFv libraries are screened for antibodies that recognize a unique protein, a protein domain, or even a protein modified by posttranslational modifications ***(4–6)***. Also, recombinant antibodies that recognize small molecules such as regulatory metabolites can be expressed. Furthermore, the function of the protein of interest can be followed in cells with different genetic backgrounds, as long as they can take up the plasmid and express the scFv-GFP fusion.

2. Materials

2.1. Plasmid

The plasmid pTRAY (Thiamine Repressible Antibody expression in Yeast) (**Fig. 1**) is designed to express scFv genes from recombinant antibody M13 phage-display libraries (Amersham Pharmacia Biotech, Baie d'Urfé, Canada; RPAS kit, cat. nos. 27-9400 and 27-9401) fused to an enhanced GFP, under the control of an attenuated *nmt1* promoter, in *S. Pombe* (*see* **Note 1**). The pTRAY plasmid is available upon request.

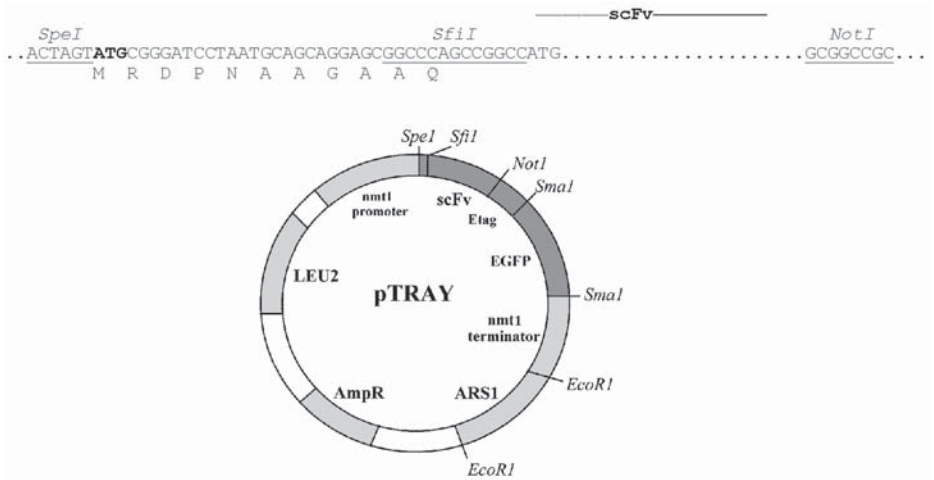

Fig. 1. Plasmid for the expression of enhanced GFP-tagged scFv proteins in *S. pombe*. Any scFv gene from the Recombinant Phage Antibody System - RPAS kit (Amersham Pharmacia Biotech) can be cloned into the pTRAY plasmid between the *Sfi*I and *Not*I restriction sites. Translational start is from an ATG immediately downstream of the *Spe*I site (*see* **Note 1**).

2.2. Culture Media, Reagents, Buffers, and Fixatives

1. Lithium acetate (0.1 *M*). The pH is adjusted to 4.9 with acetic acid. The solution is sterile-filtered and stored at room temperature.
2. PEG 4000 (Sigma-Aldrich, Oakville, Canada; cat. no. P3640) (50% w/v). The solution is difficult to dissolve and requires gentle heating to go into solution. Allow the solution to cool and sterilize by filtration. Prepare 50 mL ahead of time and store at room temperature.
3. EMM culture medium: leucine ± thiamine. EMM (Edinburgh minimal medium, from Q-BIOgene, Montreal, Canada, cat. no. 4110-032) (32 g/L) is supplemented with 100 µg/L each of adenine, uracil, histidine, and lysine. Adjust the pH to 5.5. Autoclave at 121°C for 25 min. For culture plates, add agar (20 g/L) before autoclaving. After cooling to about 60°C, add thiamine to a final concentration of 5 µg/mL.
4. Thiamine (Sigma-Aldrich, cat. no. T4625), 5 mg/mL in water, sterile-filtered and kept in the dark at 4°C.
5. YES culture medium (from Q-BIOgene, cat. no. 4110-532), 35 g/L. Autoclave at 121°C for 25 min.
6. TE: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* Ethylenediaminetetraacetic acid (EDTA).
7. Formaldehyde fixative solution (5X): Add 8.75 g paraformaldehyde (Sigma-Aldrich, cat. no. P-6148) into a 50-mL centrifuge tube with cap. Fill the tube with phosphate-buffered saline (PBS) and add 1 mL NaOH (1 *M*). Incubate at 60–65°C for 15 min with occasional shaking by inversion. Remove polymers and

other undissolved material by centrifugation at 3000*g* for 5 min or filter with Whatman no. 1 filter paper. Use the clear supernatant to fix the cells.
8. Methanol (100%; kept at –20°C); Methanol 75%, 50%, 25% (v/v) in PBS at room temperature.
9. PBS ± azide (as preservative). For a 10X PBS solution, add 87.66 g/L NaCl, 1.104 g/L $NaH_2PO_4 \cdot H_2O$, 2.413 g/L Na_2HPO_4, adjust the pH to 7.4 and sterilize by filtration. Dilute 1:9 (v/v) with sterile water for a 1X PBS solution and add Na azide to a final concentration of 1 m*M*.
10. Gelatin for coating microscope slide, 25% (w/v) in EMM-leucine. Dissolve by heating and use immediately.

3. Methods

3.1. Cell Transformation

Any *S. pombe* strain with the *leu*1-32 allele can be used to take up the pTRAY plasmid and express scFv-GFP fusions. We routinely carry out cell transformations by the lithium acetate procedure, adapted from Moreno et al. *(7)*. Other methods (e.g., electroporation) would work as well. Cells are transformed with pTRAY plasmids with and without scFv insert to compare intracellular location of the scFv-GFP fusion relative to GFP alone.

1. Start a pre-culture from a single colony in 5 mL YES medium at 30°C for 24 h (or at 25°C and up to 48 h for temperature-sensitive (ts) and/or slow-growing strains).
2. Start a 100 mL culture in YES medium from the pre-culture at a cell density of 1×10^5 cells/mL in 250-mL Erlenmeyer flask and incubate for 24 h at 30°C (or 25°C, depending on the strain) with continuous shaking (125 rpm).
3. Harvest the cells by centrifugation at 5000*g* for 5 min at room temperature.
4. Add 40 mL sterile distilled water to the cell pellet, resuspend by repeated inversions, and harvest the cells as above.
5. Repeat the cell wash with 40 mL lithium acetate solution as above, spin, and resuspend the cells in 0.5–1.0 mL lithium acetate to a density of about 1×10^9 cells/mL. Cells (0.1-mL aliquots in sterile Eppendorf tubes) are incubated for 1 h at 30°C (25°C for ts-strains). Cells sediment at this stage.
6. Add up to 2 µg plasmid DNA (pTRAY vector with and without scFv insert) in 15 µL TE and mix by gentle vortexing to resuspend the cells. Add 290 µL of PEG solution prewarmed to 30°C (25°C for ts-strains) and vortex. Incubate for 1 h.
7. Heat-shock at 42–43°C for exactly 15 min. Cool the tubes to room temperature for 10 min.
8. Harvest the cells by centrifugation (Eppendorf centrifuge at max speed for 3 min). Remove the supernatant by aspiration. Resuspend the cells in 0.5 mL sterile distilled water with the help of a sterile toothpick. Harvest the cells by centrifugation and resuspend in 0.25 mL sterile water.
9. Plate aliquots (50 µL) onto EMM-leucine + thiamine and incubate at 30°C (25°C for ts-strains) until appearance of colonies (about 4 d).
10. Re-streak 1 colony onto a fresh EMM-leucine + thiamine plate.

3.2. Cell Cultures and Expression of scFv-GFP Fusions (see Note 2)

1. Start pre-cultures from a single colony of cells transformed with a pTRAY plasmid (with and without scFv insert) in 5 mL of EMM-leu + thiamine. Expression of the scFv-GFP fusion is repressed at this point. Incubation is for 24 h at 30°C with shaking at 125 rpm.
2. Add 25 mL sterile distilled water and harvest the cells by centrifugation (5000*g* for 5 min).
3. Wash the cells to remove the extracellular thiamine at least twice with 25 mL sterile water, harvesting the cells by centrifugation each time.
4. Resuspend the cells in 5–20 mL sterile water and estimate the cell density with a cell-counting chamber.
5. Start the cultures at 1×10^5 cells/mL in EMM-leucine with and without 5 µg/mL thiamine and incubate for 24 h (*see* **Note 3**). After 24 h, cells are in early to mid-log phase and can either be fixed, or examined live to follow changes in intracellular location by time-lapse microscopy. An example of the intracellular structures observed in fixed and living cells with ectopic expression of an scFv-GFP fusion (scFv-$^{\alpha Cdc4p}$-GFP) that specifically recognizes Cdc4p, a myosin light chain, is shown in **Fig. 2**.

3.3. Microscopy (Fixed and Live Cells)

Because the antibody-GFP fusions are expressed within the cells, they can be examined immediately without fixation. Any standard fluorescent microscope with an appropriate set of filters may be used to visualize the intracellular distribution of scFv-GFP fusions. We used an Olympus IX-70 inverted microscope with 60X 1.4NA Plan-apo objective, an FITC filter set, and a RT-Slider (SPOT) CCD camera (CARSEN Scientific Imaging Group, Markham, Canada). However, we often find it convenient to fix the cells for prolonged storage (up to a month), and for co-staining the nucleus and other markers of progression through the cell cycle. For cell fixation, we used formaldehyde or methanol fixation, adapted from Moreno et al. *(7)*. The choice of fixation protocol is dependent on the structure or protein studied. It is recommended to try both protocols.

3.3.1. Methanol Fixation

1. Collect the cells in early to mid-log phase (about 40 mL) by filtration through a 100-mL Nalgene 0.22 µm filter unit under mild vacuum suction.
2. Immediately add a small volume of methanol (about 50 mL) kept at –20°C to wash the cells.
3. Disconnect the vacuum and add enough methanol to cover the cells. Place in a freezer at –20°C for 10 min. Flakes of cells form on the filter at that point.
4. Dislodge the flakes from the filter by tapping on the filter unit and transfer the suspension to a 50-mL centrifuge tube. Collect the flakes by centrifugation at 5000*g* for 5 min.

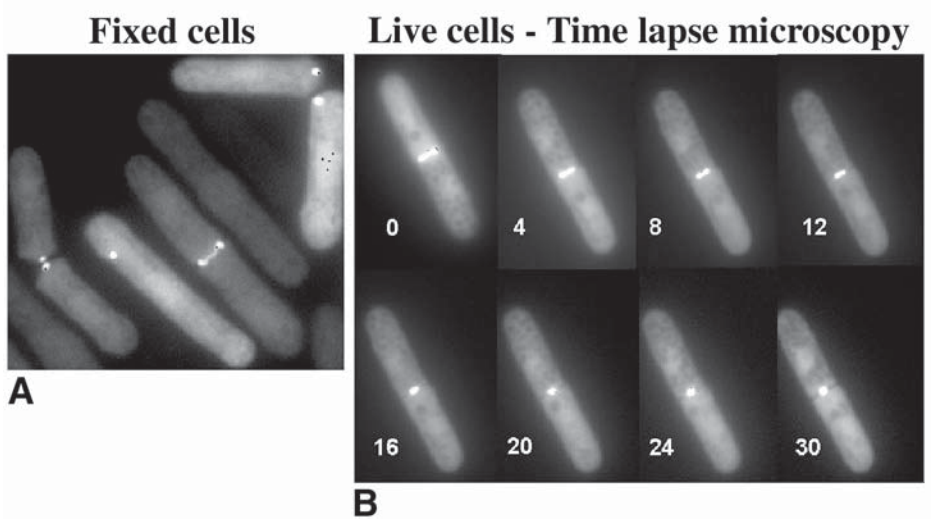

Fig. 2. Visualization of contractile ring assembly and constriction with a recombinant antibody: GFP fusion (scFv$^{\alpha Cdc4p}$-GFP) that selectively recognizes Cdc4p. Cdc4p is a contractile ring protein essential for cytokinesis in *S. pombe* ***(15,16)***. It functions as an essential light chain, binding to several myosin heavy chains ***(16)***. The figure on the left shows Cdc4p-containing structures in formaldehyde-fixed cells, as visualized by ectopic expression of scFv$^{\alpha Cdc4p}$-GFP from the pTRAY episome. The scFv$^{\alpha Cdc4p}$-GFP recognizes the contractile ring (a medial band at the center of the cells) but also Cdc4p-containing "dots" at the tip and in the middle of the cells. These "dots" contain contractile ring material that remains at the cell tip following cell division and then moves around and persists in each daughter cell for some time after division ***(2,17)***. The scFv$^{\alpha Cdc4p}$-GFP can be used also to monitor constriction of the contractile in living cells by time-lapse microscopy, as shown on the right. The numbers are times in minutes. Binding of the scFv protein to Cdc4p does not interfere with Cdc4p function, because cell growth and morphology are unaffected (not shown) and the time-course of ring constriction monitored in real time is similar to that observed by others with many different protein markers ***(2,17)***. The scFv$^{\alpha Cdc4p}$-GFP in pTRAY is available on request.

5. Discard the supernatant, add 25 mL 75% methanol/PBS, incubate for 5 min with shaking on a platform shaker. Collect the cells by centrifugation as above.
6. Repeat **step 5** with 50% methanol/PBS, followed by 25% methanol/PBS, and finally with two washes with PBS.
7. Resuspend the cells in 1 mL PBS + azide and store at 4°C.

3.3.2. Formaldehyde Fixation

1. Add 1 volume of 5X formaldehyde solution to 4 volumes of cell suspension. The formaldehyde solution is prepared just before use.
2. Incubate for 30–60 min with shaking.
3. Collect the cells by centrifugation (5000*g* for 5 min).
4. Wash the cells 3× with PBS.
5. Resuspend the cells in PBS + azide and store at 4ºC.

3.3.3. Live Cells: Time-Lapse Microscopy

With the use of antibody-GFP fusions, it is not necessary to fix the cells. A few µL of a cell suspension is squeezed between a coverslip and a microscope slide and the cells can be examined immediately. For time-lapse microscopy, it is important that the cells remain healthy and immobile for the duration of the experiment. We use cell adhesion to a semisolid gelatin surface containing growth medium.

1. A drop of heat-melted gelatin solution is spread over a microscope slide with a coverslip held at a 45° angle to form a thin layer. Do not allow this layer to dry.
2. Place a drop of cell suspension (early to mid-log growth phase) on the layer of gelatin for 5–10 min. Aspirate off to leave a thin film of cells.
3. Press a coverslip gently against the cells. The cells are then examined with a fluorescent microscope with pictures taken at regular intervals. Keep exposure to the fluorescent light as brief as possible to minimize bleaching

4. Notes

1. The vector pTRAY (**Fig. 1**) was constructed from pREP41-EGFP-C ***(8)***, obtained from Iain Hagan, University of Manchester, UK. The plasmid was modified such that any scFv sequences derived from the pCANTAB5E vector (Recombinant Phage Antibody System - RPAS Kit, Amersham Pharmacia Biotech) can be cloned between the *Sfi*I and *Not*I sites of pTRAY. Sequences encoding the E-peptide were inserted between the *Nde*I and *Sma*I sites of pREP41-EGFP-C. Both the E-tag and the GFP sequences are in reading frame for identification and expression of the scFv-GFP fusions. A 10 amino acid N-terminal extension with an initiator methionine was inserted between the *Spe*I and *Sfi*I site. The polymerase chain reaction (PCR) primers provided in the RPAS kit that are used to clone the scFv antibody into the pCANTAB5E vector introduce an ATG immediately downstream of the *Sfi*I restriction site. This ATG could be used to initiate translation of the antibody-GFP fusion from the pTRAY plasmid (**Fig. 1**). However, little to no expression was observed in yeasts in the absence of a 5' extension (that includes an initiator ATG; in bold in **Fig. 1**) inserted between the *Spe*I and *Sfi*I site of pTRAY. It could be that the hairpin structure of the *Sfi*I site impairs the translation start just downstream of this site. An alternative explanation is that the 5' extension brings the initiator ATG closer to the TATA box of the

nmt1 promoter, because translation efficiency is distance-dependent. Expression of scFv fusion proteins in *S. pombe* host strains is directed by the thiamine-repressible, attenuated *nmt1* promoter ***(9)***. Selection for pTRAY in *S. pombe* is provided by the LEU2 gene in strains with the *leu*1-32 allele.

2. Most studies in which recombinant antibodies were expressed within eukaryotic cells have focused on the ability of the antibodies to inhibit the function of a target protein, although only few have proven effective ***(10–12)***. Not all recombinant antibodies may fold correctly. Folding characteristics are an intrinsic property of the scFv amino acid sequence. Antibodies that do not fold well are rapidly degraded or form aggregates ***(13)***. However, the GFP probably assists in keeping the scFv moiety in soluble form because the GFP itself is highly soluble ***(14)***. The tendency to form aggregates appears dependent on the intracellular conditions (e.g., pH, temperature) and the level of scFv protein accumulation. With the $scFv^{\alpha Cdc4p}$-GFP (**Fig. 2**), expression levels are lower with cultures at 25°C than at 30°C and prolonged exposure to 37°C causes formation of aggregates.
3. When expressing a recombinant antibody fused to a fluorescent protein within yeast cells for protein localization, it is important that the antibody does not interfere with the function of the target antigen. Appropriate controls include examination of growth rate and morphology of cells transformed with the pTRAY plasmid with and without scFv insert, cultured in the presence (expression turned "off") and absence (expression turned "on") of thiamine. As with all *nmt1* promoter driven gene expression in *S. pombe*, there is little to no protein accumulation within the first 12–14 h. Fluorescent structures are clearly visible after 24 h in cells cultured in the absence of thiamine, but not in its presence.

Acknowledgments

This work was supported by a grant from the National Research Council of Canada to SMH.

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12

Yeast Transformation by the LiAc/SS Carrier DNA/PEG Method

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Summary

The technique for the transformation of *Saccharomyces cerevisiae* using the LiAc/SS Carrier DNA/PEG method is described. We describe a rapid method, for use when large numbers of transformants are not necessary. A high-efficiency method for the generation of large numbers of transformants is also given. A method for the transformation of plasmid libraries, which includes yeast two-hybrid applications, also is listed to aid the reader in generating transformants to effectively cover the library complexity. Finally, a protocol for transformation using a 96-well format is included for transformation applications that require it.

Key Words: *Saccharomyces cerevisiae*; transformation; DNA uptake; lithium acetate; polyethylene glycol; carrier DNA.

1. Introduction

The transformation of yeast cells after treatment with alkali cations was first reported in 1983 ***(1)***. The technique has been extensively modified in the succeeding 21 yr and the efficiency has been increased from 400 to more than 1×10^6 transformants/µg plasmid DNA. The most significant improvement came with the addition of single-stranded carrier DNA to the "Transformation Mix," increasing the efficiency to 5×10^4 transformants/µg plasmid DNA/10^8 cells ***(2)***. At this stage, the technique became known as the LiAc/SS-DNA/PEG protocol. Since then, we have modified and shortened the protocol in several ways: (1) reduction of the exposure to LiAc ***(3)***, (2) omission of TE buffer from the Transformation Mix and resuspension of transformed cells in water rather than TE ***(4)***, (3) optimized the number of cells and the concentrations of carrier DNA and plasmid DNA per transformation ***(5)***. We have also simplified the preparation of reagents for the protocol by showing that the carrier DNA need

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

not be sonicated and that LiAc and PEG solutions can be sterilized by autoclaving rather than filtration *(6)*. We have reported that the procedure can be scaled up for application to the yeast two-hybrid system *(6–9)*. We have also described the modification of the protocol for use in microtiter plates, allowing the simultaneous testing of multiple yeast strains or the assessment of several variables on the efficiency of transformation *(7,10)*. Yeast cells can also be transformed by electroporation *(11,12)*, by biolistics *(13)*, after treatment with glass beads *(14)*, and after conversion to spheroplasts *(15,16)*. We are most familiar with the LiAc/SS-carrier DNA/PEG method and focus on it in this chapter. For a review of other methods of yeast transformation, see Gietz and Woods *(17)*.

The Rapid LiAc/SS-carrier DNA/PEG Transformation Protocol is used to introduce a specific plasmid into a specific strain of yeast using with the aim of recovering and analyzing a small number of transformants. The High-Efficiency Transformation Protocol can be employed to screen multiple equivalents of yeast and other genomes for plasmids that complement a specific mutation. It can also be used to transform a particular yeast strain with an integrating plasmid or oligonucleotide *(18,19)*, to simultaneously transform a yeast strain with two different plasmids *(20)*, or to transform a plasmid library into a two-hybrid yeast strain. The Library Screen Transformation Protocol is used to generate the large numbers of transformants required to screen eukaryotic cDNA libraries that typically have a complexity of over 1×10^6 clones. This protocol can also be used for two-hybrid and similar screens *(21,22–24)*. We also include protocols for Microtiter Plate Transformation. These protocols can be used to transform plasmid libraries in a 96-well format into a single yeast strain, to transform a large number of strains in one operation, or to optimize the conditions for the transformation of one or more strains by the Rapid and High-Efficiency protocols.

2. Materials

2.1. General Equipment

1. A microtiter plate rotor is required for the Microtiter Plate transformation protocols.
2. A 96-prong replicator (Fisher Scientific, Ottawa, ON, cat. no. 05-450-9) and an eight-channel micropipettor (Eppendorf™, Westbury, NY, or TiterTek™, Huntsville, AL) are required for the Microtiter Plate Transformation protocols.
3. A platform to secure microtiter plates on a rotary shaker. One can be made from 1/4 in. plywood/plexiglass by cutting out microtiter plate size rectangles. The plates (plus lids) should fit the slots with as little play as possible. The platform can be secured to the shaker with machine screws, small C clamps, or large bulldog clips. The lids should be left loose on the plates.

Table 1
YPAD Medium

Ingredients	YPAD Agar	YPAD Broth	2X YPAD Broth
Bacto YPD Agar	50 g	—	—
Bacto YPD Broth	—	40 g	80 g
Adenine hemisulphate	80 mg	80 mg	80 mg
Distilled/deionized water	800 mL	800 mL	800 mL

2.2. Media

1. YPAD (Yeast Extract-Peptone-Adenine-Dextrose) Medium: This medium is used for routine growth of yeast strains; adenine is added to decrease the selective advantage of *ade2* to *ADE2* reversions. We use double-strength YPAD broth, 2X YPAD, to re-grow cultures to log phase before transformation. Recipes for YPAD and 2X YPAD are given in Woods and Gietz *(**8**)*; alternatively, commercial formulations of YPD agar (Bacto YPD Agar) and broth (Bacto YPD Broth) media can be obtained from Becton Dickinson Microbiology Systems (Becton Dickinson, Sparks, MD). These media should be supplemented with adenine hemisulphate as above. *See* **Table 1**.
   Volumes of 800 mL can be made up and autoclaved in 1-L Pyrex™ medium bottles or other suitable 1-L containers. Add the Bacto YPD Agar to the water and stir with a magnetic stir bar on a stirring hot plate. Continue stirring and boil for 1 min to ensure that the agar is dissolved. Autoclave the medium for 15 min and allow it to equilibrate to 60°C in a water bath before pouring it into petri dishes. This volume of medium is sufficient for about 30 plates. Allow the plates to dry overnight and then store them in plastic sleeves in the dark at 4°C. Dissolve YPAD and 2X YPAD broth on a stirring hot plate; dispense in 100- or 200-mL aliquots and autoclave for 15 min. Store in the dark at 4°C.
2. SC (Synthetic Complete) Selection Medium. SC selection medium is made by adding a mixture of amino acids, purines, pyrimidines, and vitamins to Difco Yeast Nitrogen Base. Specific components of the mixture are omitted in order to select for the genetic marker carried by the plasmid. Thus SC minus Ura, SC minus Trp, SC minus His, SC minus Leu, and SC minus Ade lack uracil, tryptophan, histidine, leucine, and adenine, respectively, and are used to select for plasmids carrying the selectable markers *URA3*, *TRP1*, *HIS3*, *LEU2,* and *ADE2*. *See* **Table 2**.
   Add the ingredients to the water in a 1-L Pyrex medium bottle and stir with a magnetic stir bar on a stirring hot plate at room temperature. Adjust the pH to 5.6 with 1 *N* NaOH; turn on the heater and bring the medium to a boil for 1 min to dissolve the agar. Autoclave the medium for 15 min and allow it to equilibrate to 60°C in a water bath before pouring it into Petri dishes. This medium is light-sensitive; plates should be dried in the dark at room temperature for 1 or 2 d and then stored in sealed bags in the dark at 4°C. Liquid medium should also be stored in the dark at 4°C.

Table 2
Synthetic Complete Medium

Ingredient	SC selection medium
Difco Yeast Nitrogen Base w/o amino acids	5.4 g
Amino acid mix	1.6 g
Glucose	16.0 g
Difco Bacto agar[a]	12.0 g
Distilled/deionized Water	800.0 mL

[a]Omit the agar to make liquid SC selection medium.

Table 3
Amino Acid Mix

Compound	Quantity
Adenine SO4	**0.5 g**
Arginine	2.0 g
Aspartic Acid	2.0 g
Glutamic Acid	2.0 g
Histidine HCl	**2.0 g**
Inositol	2.0 g
Isoleucine	2.0 g
Leucine	**4.0 g**
Lysine HCl	2.0 g
Methionine	2.0 g
Phenylalanine	2.0 g
Serine	2.0 g
Threonine	2.0 g
Tryptophan	**2.0 g**
Tyrosine	2.0 g
Uracil	**2.0 g**
Valine	2.0 g
p-Aminobenzoic acid	0.2 g

3. Amino Acid Mix. Mix the following ingredients ***(23)*** in a plastic container by shaking thoroughly with two or three glass marbles. The compounds omitted in specific SC selection media are in bold type. *See* **Table 3**.

2.3. Solutions

1. Lithium acetate (1.0 *M*). Add 5.1 g of lithium acetate dihydrate (Sigma Chemical Co. Ltd., St. Louis, MO, cat. no. L-6883) to 50 mL of water in a 100-mL Pyrex medium bottle, stir until dissolved, autoclave for 15 min, and store at room temperature.

Table 4
Transformation Mix

Component	Volume
PEG 3500 (50% [w/v])	240 µL
LiAc 1.0 *M*	36 µL
SS carrier DNA (2.0 mg/mL)[a]	50 µL
Plasmid DNA (100 ng) plus water (distilled/deionized)	34 µL
Total volume (excluding cells)	360 µL

[a]Vortex mix the carrier DNA before pipetting it.

2. PEG MW 3350 (50% [w/v]). Add 50 g of PEG 3350 (Sigma Chemical Co. Ltd., cat. no. P-3640) to 30 mL of distilled/deionized water in a 150-mL beaker. Dissolve on a stirring hot plate with medium heat and then cool to room temperature. Make the volume up to 100 mL in a 100-mL measuring cylinder, cap the cylinder with Parafilm™, and mix by inversion. Transfer the solution to a glass storage bottle and autoclave for 15 min. The polyethylene glycol (PEG) can be stored at room temperature. The bottle must be securely capped bottle to prevent evaporation, which will increase the concentration of PEG in the transformation reaction and severely reduce the yield of transformants.
3. Single-stranded carrier DNA (2.0 mg/mL). Dissolve 200 mg of salmon sperm DNA (Sigma Chemical Co. Ltd., cat. no. D-1626) in 100 mL of TE (10 m*M* Tris-HCl, 1 m*M* Na_2 EDTA, pH 8.0) on a stir plate overnight at 4°C. Dispense 20 samples of 1.0 mL into 1.5-mL microcentrifuge tubes and the remainder in 5 mL samples in 15-mL screw-capped plastic centrifuge tubes and store at –20°C. Denature the carrier DNA in a boiling water bath for 5 min and chill immediately in an ice/water bath before use. Denatured carrier DNA can be boiled three or four times without loss of activity.
4. Transformation Mix All three transformation protocols use the same basic Transformation Mix (T Mix). The recipe below is for the transformation of 1×10^8 cells; the volumes can be amended as appropriate for larger and smaller numbers of cells. T Mix can be made up in bulk and kept in ice/water until required. The highest *transformation efficiencies* (transformants/µg plasmid DNA/10^8 cells) are obtained with 100 ng plasmid DNA but the *yield* (number of transformants) will be increased if more plasmid is used. *See* **Table 4**.

3. Methods

3.1. Rapid Transformation Protocol

D 1

1. Inoculate a 2 cm^2 patch of the yeast strain onto YPAD agar and incubate overnight at 30°C (*see* **Note 1**).

D 2

2. Boil a tube of carrier DNA in a boiling water bath for 5 min and chill immediately in ice/water. We suggest that you do this first, otherwise you will have to put subsequent steps on hold for 10 to 15 min.
3. Scrape a 50 µL blob of yeast from the YPAD plate and suspend the cells in 1.0 mL of sterile water in a 1.5-mL microcentrifuge tube. The suspension will contain about 5×10^8 cells.
4. Pellet the cells at top speed in a microcentrifuge for 30 s and discard the supernatant.
5. Add 360 mL of T Mix to the cell pellet. Resuspend the cell pellet by vortex mixing briskly. For a single transformation, the ingredients should be added in the order listed and mix vigorously.
6. Incubate the tube in a water bath at 42°C for 20–180 min (*see* **Notes 2** and **3**).
7. Microcentrifuge the transformation tube at top speed for 30 s and remove the T Mix with a micropipettor.
8. Pipet 1.0 mL of sterile water into the transformation tube. Stir the pellet with a sterile micropipet tip to resuspend the cells and then vortex mix vigorously.
9. Pipet 10 and 100 µL samples of the cell suspension onto plates of appropriate SC selection medium. The 10 µL samples should be pipetted into 100 µL puddles of sterile water. Transformants can be isolated after incubation at 30°C for 3 or 4 d.

3.2. High-Efficiency Transformation Protocol

D 1

1. Inoculate your yeast strain into 5 mL of liquid medium (2X YPAD or appropriate SC selection medium) and incubate overnight on a rotary shaker at 200 rpm and 30°C. Place a bottle of 2X YPAD and a 250-mL culture flask in the incubator as well.

D 2

2. Determine the titer of the yeast culture.
   a. Pipet 10 µL of cells into 1.0 mL of water in a spectrophotometer cuvet, mix thoroughly by inversion, and measure the OD at 600 nm (a suspension containing 1×10^6 cells/mL will give an OD_{600} of 0.1). Remember to multiply by the dilution factor to determine the titre in the cell culture.
   b. Pipet 100 µL of suspension into 900 µL of sterile water in a microcentrifuge tube and mix thoroughly. Deliver 10 µL of this dilution onto the counting grid of an improved Neubauer haemocytometer, put the coverslip in place, wait several minutes for the cells to settle, and count the number of cells in the 25 large grid squares. Multiply this number by 10,000 to obtain the titer in the diluted suspension. Remember to multiply by the dilution factor to determine the titer in the cell culture (*see* **Note 4**).
3. Add 2.5×10^8 cells to 50 mL of the pre-warmed 2X YPAD in the pre-warmed culture flask. The titer will be 5×10^6 cells/mL.

4. Incubate the flask in the shaking incubator at 30°C and 200 rpm until the cell titer is at least 2×10^7 cells/mL. This should take about 4 h.
5. Denature a 1.0-mL sample of carrier DNA in a boiling water bath for 5 min and chill immediately in an ice/water bath.
6. Harvest the cells by centrifugation at 3000*g* for 5 min, wash the pellet twice in 25 mL of sterile water, and resuspend the cells in 1.0 mL of sterile water.
7. Transfer the cell suspension to a 1.5-mL microcentrifuge tube, centrifuge for 30 s, and discard the supernatant.
8. Resuspend the cells in 1.0 mL of sterile water and pipet samples of 10^8 cells into 1.5-mL microfuge tubes, one for each transformation. Centrifuge at top speed for 30 s and remove the supernatant.
9. Make up sufficient T Mix (*see* **Subheading 2.**, Materials) for the planned number of transformations plus one extra. Keep the T Mix in ice/water.
10. Add 360 µL of T Mix to each transformation tube and resuspend the cells by vortex mixing vigorously.
11. Place the tubes in a floating rack and incubate them in a water bath at 42°C for 40 min (*see* **Note 5**).
12. Microcentrifuge the tubes at top speed for 30 s and remove the T Mix with a micropipettor.
13. Pipet 1.0 mL of sterile water into the transformation tube. Stir the pellet with a sterile micropipet tip to resuspend the cells and then vortex mix vigorously.
14. Plate appropriate dilutions of the cell suspension onto SC selection medium. With many strains, you can anticipate 2×10^6 transformants/µg plasmid DNA/10^8 cells. If you used 100 ng of plasmid per transformation this will result in 2×10^5 transformants per tube or 200 per 1.0 µL. Dilute 10 µL of the suspension into 1.0 mL of water and plate 10 and 100 µL samples onto two plates each. The 10 µL samples should be pipetted directly into 100 µL puddles of sterile water on the SC selection medium (*see* **Note 6**).
15. Incubate the plates at 30°C for 3–4 d and count the number of transformants.
16. Calculate the transformation efficiency and yield of transformants (*see* **Note 7**).

3.3. The Library Screen Transformation Protocol

This protocol is used to generate the large numbers of transformants required to screen complex plasmid libraries. Before attempting such a screen, it is advisable to use the High-Efficiency protocol to test the effects of increasing plasmid DNA on transformation efficiency and transformation yield. Yield is the more important parameter in these circumstances; scale up 30- or 60-fold with an appropriate plasmid concentration to obtain the number of transformants required (*see* **Note 7**). We have set out a protocol for a regular large-scale screen, specific requirements for the two-hybrid and similar screens are shown in **Notes 8–12**. Additional information for a yeast two-hybrid screen can be found within this volume ***(24)***.

D 1

1. Inoculate your yeast strain into 50 mL of 2X YPAD in a 250-mL flask. Incubate at 30°C overnight on a rotary shaker at approx 200 rpm. Warm 200 mL (30×) or 400 mL (60×) of 2X YPAD broth and a culture flask (500 mL, 30×; 1000 mL, 60×) overnight at 30°C (two-hybrid screen; *see* **Note 11**.)

D 2

2. Determine the titer of the culture. Transfer the volume containing 6.25×10^8 cells (30× scale-up) or 1.25×10^9 cells (60× scale-up) into 50-mL centrifuge tubes and pellet the cells. Resuspend the pellet(s) in warm 2X YPAD broth and transfer to the culture flask(s). Add sufficient 2X YPAD broth to bring the final cell titer to 5×10^6/mL (two-hybrid screen; *see* **Note 12**.)
3. Incubate the flask at 30°C and 200 rpm until the cells have undergone two divisions. This will take at least 4 h.
4. Boil the SS carrier DNA (30×, 2.0 mL; 60×, 3.5 mL) for 5 min and chill in ice/water.
5. Make up appropriate volumes of T Mix and keep in ice/water. *See* **Table 5**.
6. Harvest the cells by centrifugation and resuspend them in one fifth of the culture volume of sterile water. Centrifuge and wash the cells again in the same volume of water. Centrifuge and discard the supernatant.
7. Pipet the T Mix onto the cell pellet and suspend the cells by vortexing the tube vigorously.
8. Incubate the cell suspension at 42°C for 60 min. Mix the contents of the tube by inversion at 5-min intervals to ensure temperature equilibration.
9. Centrifuge at 3000*g* for 5 min. Pour off the T Mix, centrifuge again, and remove the remainder of the T Mix with micropipettor.
10. Resuspend the cells in sterile water (30×, 20 mL; 60×, 40 mL) and spread 400 µL samples onto 150-mm plates of SC selection agar (30×, 50 plates; 60×, 100 plates) (two-hybrid screen; *see* **Note 13**).
11. Incubate the plates at 30°C for 4–7 d and count and recover transformants.

3.4. Microtiter Plate Transformation Protocols

We have adapted the Rapid and High-Efficiency Protocols for the transformation of yeast cells in 96-well microtiter plates with round bottoms (*see* **Note 14**). These protocols can be tailored for many different purposes (*see* **Note 15**). For large numbers of transformations, we use a 96-prong replicator and 150-mm Petri dishes of medium; however, we find it convenient to use a custom-made 8×8 well replicator (lacking the four-corner prongs) and 100-mm Petri dishes for up to 60 transformations. The T Mix for these protocols is prepared without PEG; it is less viscous than regular T Mix and makes is easier to resuspend the cell pellet. The PEG is added after the cell pellets have been resuspended.

Table 5
Transformation Mix Volumes

Ingredients	30×	60×
PEG 50% (w/v)	7.2 mL	14.4 mL
LiAc 1.0 *M*	1.08 mL	2.16 mL
SS carrier DNA (2 mg/mL)	1.5 mL	3.0 mL
Plasmid DNA + water	1.02 mL	2.04 mL
Total volume	10.8 mL	21.6 mL

3.4.1. Agar Plate Protocol

D 1

1. Dip the prongs of a replicator into a dish of 95% ethanol and sterilize them by passing them through a Bunsen flame.
2. Set the replicator "prongs up" in a beaker and press the agar surface of a plate of YPAD onto the prongs so that all of them make an imprint.
3. Use an inoculating loop or sterile flat toothpicks to patch the yeast strain(s) onto the imprints. Orient this "master" plate(s) by marking the bottom and incubate overnight at 30°C.

D 2

4. Pipet 150 µL samples of sterile water into the wells of a microtiter plate.
5. Sterilize a replicator and set it "prongs up" in a beaker.
6. Invert the "master" plate and align the patches of yeast with the tips of the prongs. Lower the plate onto the prongs, making sure that all of the patches of yeast make contact. Move the plate very gently in small circles to transfer cells to the prongs. Remove the "master" plate and inspect the prongs. You can use a toothpick or inoculating loop to adjust the quantity of cells on individual prongs if necessary.
7. Lower the replicator into the microtiter plate and agitate it up and down to suspend the cells. This should result in about 1×10^7 cells per well. Repeating the transfer process will increase the number of cells. Mark the orientation of the microtiter plate.
8. Centrifuge the plate with an appropriate balance plate for 10 min at 3500 rpm in a microtiter plate rotor.
9. Remove the supernatant from the wells by aspiration with a sterile micropipet tip attached to a vacuum line. Do not touch the cell pellet with the tip! It is possible to shake the water out of the wells into a sink, but it is best to practice this operation before a critical experiment.
10. Boil a tube of carrier DNA (2 mg/mL) for 5 min and chill in ice/water.
11. Prepare T Mix minus PEG. The volumes listed are for one transformation (one well). Make sufficient for 100 transformations if you intend to use all 96 wells.

Table 6
Microtiter Transformation Mix

Component	One well
LiAc 1.0 *M*	15.0 µL
Carrier DNA (2 mg/mL)	20.0 µL
Plasmid DNA (20 ng) + water	15.0 µL
Total volume	50.0 µL

You can use more or less than the listed amount of plasmid DNA. Keep the T Mix minus PEG on ice. *See* **Table 6**.

12. Pipet 50 µL T Mix minus PEG into each well. Clamp the plate on a rotary shaker and shake it for 2 min at 400 rpm to resuspend the cell pellets.
13. Pipet 100 µL PEG 3350 (50% [w/v]) into each well. Shake the plate for 5 min at 400 rpm and check that the cell suspensions are homogeneous.
14. Put the microtiter plate into a ZipLoc™ sandwich bag or seal it with Parafilm and incubate it at 42°C for 1–4 h (*see* **Note 16**).
15. Centrifuge the microtiter plate for 10 min at 3500 rpm and remove the T Mix by aspiration.

Quantitative Sampling

16. Pipet 100 µL of water into each well and shake the plate at 400 rpm for 5 min to resuspend the cells. Pipet 5 µL samples into 100 µL puddles of water on plates of SC selection medium.

Qualitative Sampling

17. Pipet 50 µL of water into each well and resuspend the cells as in **step 16**. Sterilize a replicator and use it "prongs down" to print samples (approx 10 µL) onto plates of SC selection medium. Additional samples can be overlaid if necessary.
18. Incubate the plates at 30°C for 2–4 d and recover and/or count the transformants.

3.4.2. Liquid Culture Protocol

The yeast culture is grown overnight and regrown for two divisions as in the High-Efficiency Transformation Protocol. The cells of the regrown culture are harvested, washed, and resuspended in water and the cell titer determined as described in the High-Efficiency Transformation Protocol.

1. Adjust the titer of the cell suspension to 4×10^8 cells/mL and dispense 100 µL samples of the suspension into the wells of the microtiter plate.
2. Continue from **step 6** of **Subheading 3.4.1.,** Agar Plate Protocol, but increase the amount of plasmid to 100 ng/transformation.
3. Seal and incubate the plates at 42°C for 60 min.

4. Sample the wells by plating or replica plating onto SC selection medium.
5. Incubate the plates at 30°C for 2–4 d and recover and/or count the transformants.

4. Notes

1. This protocol can be used to transform cells that have been stored in a refrigerator or at room temperature. The yield will be reduced but there will generally be sufficient transformants of the desired genotype.
2. Incubation at 42°C for 20 min will result in several thousand transformants per tube. With many yeast strains, extending the duration of this incubation can increase the yield of transformants significantly. We have obtained 1×10^5 transformants/µg plasmid after 60 min incubation and $>1 \times 10^6$/µg plasmid DNA after 180 min.
3. The addition of dimethylsulfoxide (DMSO) to the T Mix increases the yield of transformants with some strains. For example, when strain Y190 was transformed by the Rapid Protocol the yield of transformants increased over 10-fold when 5% DMSO was added to the T Mix and incubation at 42°C was extended to 180 min.
4. The counting grid is made up of 25 large squares bounded by triple lines; each large square is subdivided into 16 small squares bounded by single lines. The 25 large squares cover an area of 1.0 mm^2 and the depth beneath the coverslip is 0.1 mm. The total volume of the counting area is 0.1 µL.
5. The addition of 1% DMSO to T Mix in the High-Efficiency protocol increases the number of transformants about twofold *(**10**)*.
6. These calculations are appropriate for YEp, YRp, or YCp library plasmids. If you are transforming with an integrating plasmid (YIp), a linear construct or an oligonucleotide, plate 200 µL onto each of five plates of SC medium.
7. The transformation efficiency is the number of transformants/1 µg plasmid DNA/ 10^8 cells. If you used 100 ng of plasmid DNA to transform 1×10^8 cells and obtained 500 colonies by plating 100 µL of a 10 µL into 1.0 mL dilution of the resuspended cells, then:

$$\text{Transformation Efficiency} = 500 \times 1000 \text{ (plating factor)} \times 10 \text{ (plasmid factor)} \times 1 \text{ (cells/transformation} \times 10^8\text{)}.$$

$$\text{Transformation Efficiency} = 5 \times 10^6 \text{ transformants/1.0 µg plasmid/}10^8 \text{ cells}.$$

The total yield of transformants is this instance would be the plate count multiplied by the dilution factor = $500 \times 1000 = 5 \times 10^5$ transformants. Increasing the amount of plasmid DNA per transformation reduces the efficiency, but increases the yield of transformants, as shown in **Table 7**.
In this example, the most efficient scale up would be to use 1.0 µg plasmid per 10^8 cells. A 30× scale-up would require 30 µg plasmid DNA and should yield $30 \times 1.55 \times 10^6 = 4.6 \times 10^7$ transformants.

8. Two-hybrid screens typically require the transformation of "bait" and "prey" plasmids into a specific yeast strain. The genotypes of suitable yeast strains and procedures for the construction and testing of fusion plasmids can be found in Gietz et al. *(**6**)*.

Table 7
Transformation Efficiency vs Transformation Yield

Plasmid DNA (µg)	Transformation Efficiency ($\times 10^6$)	Transformant Yield ($\times 10^6$)
0.1	2.55	0.25
1.0	1.55	1.55
5.0	0.89	1.77
10.0	0.19	1.89

9. A two-hybrid screen involves the transformation of the "prey" plasmid library into yeast cells carrying the "bait" plasmid. Use the High-Efficiency Protocol to transform the yeast strain contain the "bait" plasmid with a range from 100 ng to 10 µg of the "prey" plasmid library to determine the appropriate scale-up factor (*see* **Note 7**).
10. The "bait" plasmid and the "prey" plasmid library can be co-transformed into the yeast strain in a single operation. The high-transformation efficiencies obtained with these protocols can result in up to 40% of the transformed yeast cells containing both plasmids ***(20)***. Co-transformation may be necessary if the "bait" plasmid affects the growth or viability of your yeast strain.
11. Inoculate the strain carrying "bait" plasmid into liquid SC selection medium. Use 50 mL medium in a 250-mL flask for a 30× scale-up and 100 mL medium in a 500-mL flask for a 60× scale-up.
12. The strain carrying the "bait" plasmid can be cultured in 2X YPAD for the two divisions prior to transformation without significant loss of the plasmid, but must be maintained on SC selection medium to retain the plasmid.
13. In a two-hybrid screen, the yeast strain contains a reporter gene that is activated by interaction of the protein products of the "bait" and "prey" plasmids. Details of the selection and detection of reporter gene activation are given in Gietz et al. ***(6)*** and Gietz and Woods ***(9)***.
14. Microtiter plates can be purchased sterile and discarded after use or they can be washed and sterilized by UV irradiation and used again.
15. The Microtiter Plate Protocols can be adapted for a number of purposes.
    a. Many different yeast strains can be grown on a master plate, sampled with a replicator into the wells of a microtiter plate, and tested for transformation efficiency with a single plasmid.
    b. A single strain can be transformed with many different plasmids (e.g., a plasmid library in a 96-well format).
    c. Many yeast strains can be grown on a master plate, transferred to wells containing 150 µL of 2X YPAD, regrown in sealed plates on a shaker at 200 rpm, and then transformed *in situ* with a single plasmid.
    d. One or more strains can be tested for response to variation in the composition of the T Mix.
    e. One or more strains can be tested for response to variation in the duration of incubation at 42°C.

16. After incubation at 42°C for 60 min, we have obtained an efficiency of 2×10^5 and a yield of 570 transformants per well; extending the incubation to 4 h resulted in an efficiency of 3.9×10^6 and 6200 transformants per well.

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13

Mutagenesis

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Summary

To identify new genes in an organism, a genetic approach can be used to screen for mutations that display a particular phenotype. Genotoxic agents, such as ultraviolet (UV) light, ionizing radiation, or chemicals can be used to randomly induce DNA lesions in the genome. Most efficient mutagenesis occurs when a mutagen confers a high frequency of mutations with low lethality, in the range of 10 to 50% survival. These mutations can be in the form of frameshifts, deletions, or rearrangements. To initiate a mutagenesis, a fresh subculture of cells grown into log phase is collected, washed, and resuspended in potassium phosphate buffer. The mutagen is added to the culture for a predetermined time, deactivated, and washed from the cells. The cells are allowed to recover from the treatment by incubating in liquid or on solid medium. Mutants can be isolated by screening individual colonies or by using direct selection of cells from the mutagenized cell population.

Key Words: Yeast; mutagenesis; mutagen; genetic screen; method.

1. Introduction

The genetic approach to identifying new genes in the cell is to create mutants that display a particular phenotype. This strategy allows the researcher to examine the entire genome for genes of interest. DNA lesions can arise naturally or in the presence of a variety of genotoxic substances such as UV light, ionizing radiation, or chemicals. The best method to introduce mutations into the genome is to carry out a mutagenesis experiment using a mutagen that confers a high frequency of mutations with low lethality. Most commonly used in vivo mutagenesis protocols are based on mutations that produce base-pair substitutions; however, mutagens that introduce frameshifts, deletions, or rearrangements can also be used.

Saccharomyces cerevisiae is a model organism to study higher eukaryotes. The *S. cerevisiae* life cycle consists of both haploid and diploid states.

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

Manipulating a haploid genome through the use of a mutagenesis assay allows one to directly observe the phenotype of a particular mutation. When performing a mutagenesis experiment, it is important to select a strain that will meet specific experimental needs. Because the mutagenesis process is random and more than one mutation may be introduced into the genome, it is necessary that the mutation of interest can be subsequently placed in a nonmutagneized genetic background. This can be readily accomplished by repeated crossing to an isogenic strain of opposite mating type. The strain must therefore perform satisfactorily in mating and sporulation experiments.

The choice of mutagen used in the assay will depend on the yeast strain and type of mutation to be introduced. The alkylating agents *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) and ethyl methanesulfonate (EMS) are widely used for mutagenesis. DNA alkylating agents are electrophilic compounds that react with organic macromolecules by donating alkyl groups to the bases of the DNA molecule. Alkylating agents can bind several sites on DNA molecules by nucleophilic substitutions of S_N1- and S_N2-type reactions, with the most common binding sites under physiological conditions being oxygen or nitrogen in genomic DNA. These alkyl groups may distort the DNA helix, hindering replication and transcription, and frequently cause the incorporation of an incorrect base pair ***(1)***. MNNG is mainly involved in S_N1 reactions at oxygen molecules and is highly specific in the mechanism of action, producing mainly O^6-methylguanine lesions resulting in G-C to A-T transitions ***(2,3)***. A second lesion, O^4-methylthymine, is incorporated at a low frequency resulting in a T-A to C-G transition ***(4)***. EMS interacts with DNA molecules mainly through S_N2 reactions. This type of reaction will result primarily in base-pair substitutions ***(3)***. Exposure to UV radiation at approx 254 nm is another method of inducing mutations. It causes adjacent pyrimidines to become covalently linked, resulting in cyclobutane pyrimidine dimers and pyrimidine-pyrimidone 6-4 photoproducts ***(4)***. Other mutagens, such as ICR-191 and 4-nitroquinoline-*N*-oxide (4-NQO), can also be used to induce random mutations. ICR-191 is a intercalating agent and induces +1 frameshift mutations, whereas 4-NQO causes bulky DNA adducts ***(1)***.

The optimal dose of a mutagen usually results in 10–50% survival. This gives the highest proportion of mutants per treated cell while avoiding problems such as multiple mutations ***(5)***. Treatment of cells with a mutagen can be adapted to suit the needs of the experiment by either using a dose response to the mutagen, or by treating the cells over a time-course. By using a dose response, one is able to treat the cells for a fixed length of time with varying doses of the mutagen to achieve the optimal incorporation of mutations. A time-course treatment uses a fixed dose of a mutagen with treatment time as the variable. To determine the survival of the yeast strain when treated with a

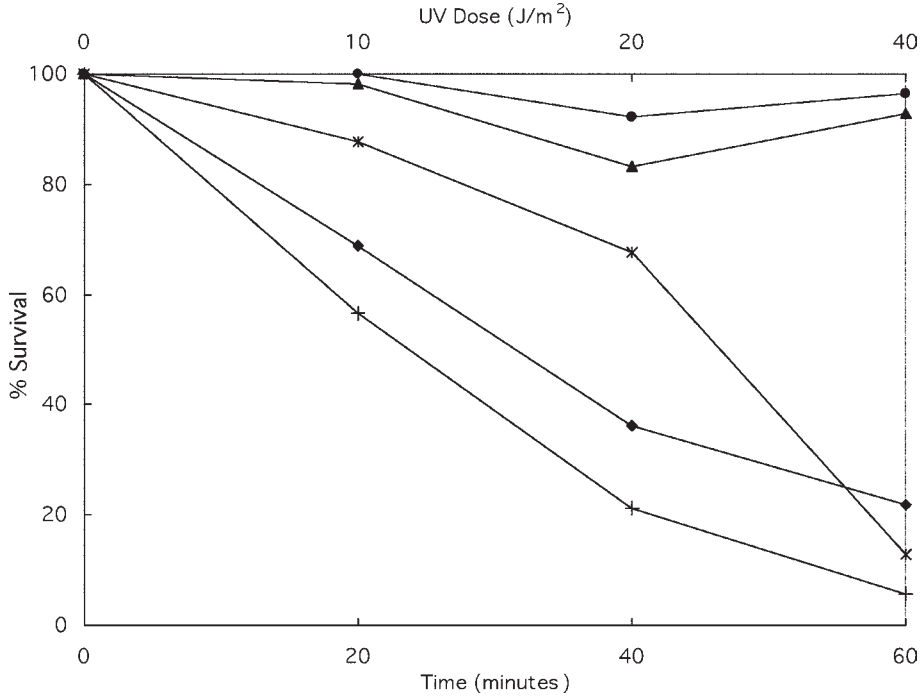

Fig. 1. Survival curves for different mutagenic doses. Strain used in treatments: RKY2672 (Mat**a** *his3Δ200 ura3-52 leu2Δ1 trp1Δ63 ade2Δ1 ade8 hom3-10 lys2ΔBgl*). Treatments were done over a time-course except for (★) UV, which was done over a dose range in J/m^2. Doses are as follows: (◆) EMS 3%, (+) MNNG 10 µg/mL, (▲) ICR-191 50 µg/mL, and (●) 4-NQO 5 µg/mL. The cultures were plated on YPD and incubated for 3 d at 30°C.

mutagen, a survival curve can be generated. By treating the cells with a mutagen over a time-course and plating for surviving cells, the percentage of survival can be determined. The optimal dose of a mutagen can then be determined from the curve (*see* **Fig. 1**).

Not all mutagens will incorporate mutations into a genome at equal frequencies. To optimize the mutagenesis, one should determine the mutation frequency of different mutagens in the yeast strain. Most laboratory strains carry genes that can be used to measure the incorporation of mutations. For example, the *CAN1* gene, which confers sensitivity to the drug canavanine in arginine prototrophs, can be used to calculate the mutation frequency. When mutations are introduced into the *CAN1* gene, the cells become resistant to canavanine ***(6)***, and colonies formed on canavanine plates can be used to calculate the frequency of forward mutations introduced by the mutagen (*see* **Table 1**). By

Table 1
Induced Can^R Forward Mutation Frequencies in RKY2672 by Different Mutagens

Drug	Dose[a]	Mutation frequency
UV	40 J/m^2	3.81×10^{-5}
EMS	3%	9.15×10^{-6}
MNNG	10 µg/mL	1.48×10^{-5}
ICR-191	50 µg/mL	2.25×10^{-6}
4-NQO	5 µg/mL	1.47×10^{-5}

[a]The cells were exposed to each chemical mutagen for 40 min, whereas UV treatment was a single dose at 40 J/m^2.

treating the yeast cells with a mutagenic dose that allows for 10 to 50% survival and plating on canavanine medium and rich medium the forward mutation frequency can be calculated using the formula

Number of Mutant Colonies/Total Number of Colonies on Nonselective Plates

Other markers, if present in the host strain, can also be used to calculate mutation frequencies. For example, the *URA3* gene has been reported for use in forward mutation assays. This marker is used in conjunction with 5-flouroorotic acid to select for uracil auxotrophs ***(7)***. Reversion systems require specific mutations and thus are not as common for determining the mutation frequency for a particular mutagen. Nevertheless, laboratory strains often carry revertable auxotrophic mutant alleles that can be used to determine the reversion frequency.

Once the optimal dose has been determined, the cells can be mutagenized. Mutants are isolated by screening individual colonies from the mutagenized cell population. Enrichment procedures, which increase the proportion of mutants, can be used to reduce this labor. For example, inositol starvation can be used to temporarily prevent mutant, but not nonmutant, growth and allow for the selective killing of growing cells ***(8)***. Alternatively, the antibiotic nystatin can be used to select specific auxotrophic mutants after mutagen treatment ***(9)***.

The desired mutation can be recovered either by screening individual colonies or by using direct selection of cells containing the desired mutation. To screen for mutations, the cells will be diluted and plated on rich medium to allow all viable cells to grow. Each individual colony will be recovered and screened under conditions that will select the desired phenotype. This method is labor-intensive and may require screening several thousand colonies. By

direct selection of mutants, this step of individually screening colonies can be eliminated. Direct selection can be used when a desired phenotype can be positively selected by plating under the appropriate conditions. For example, selection of drug-resistant mutants can be accomplished by plating the cells on medium containing an appropriate dose of the drug. A selection protocol allows only the mutants to grow; thus one is able to plate 10^5 cells per plate, increasing the efficiency of mutant recovery. The type of mutant being recovered in the mutagenesis will dictate the amount of time and materials needed in each experiment.

2. Materials

1. Water for solutions and media should be distilled and deionized (ddH_2O).
2. YEPD medium: 1% (w/v) yeast extract, 2% (w/v) bacto-peptone, and 2% (w/v) dextrose, dissolved in water, and autoclaved at 15 psi/121°C for 15 min. Liquid medium can be solidified using 2% (w/v) bacto-agar. Store liquid medium at room temperature and solidified medium at 4°C for up to 3 mo.
3. 50 m*M* Potassium phosphate buffer in ddH_2O: Make at pH 7.0.
4. MNNG: MNNG can be purchased from Sigma (St. Louis, MO). The MNNG solution should be made in a fume hood with the window lowered as much as possible. Care should be taken to avoid contact with, or inhalation of, the MNNG powder. Dispense 10 mg of MNNG into a capped, pre-weighed glass vial. Reweigh and add a sufficient volume of acetate buffer to bring the concentration to 1 mg/mL. MNNG should be used immediately or dispensed into Eppendorf tubes for storage at –20°C. Each stock tube of MNNG should only be used once and thawed on ice immediately before use. MNNG is light-sensitive and should therefore be stored in the dark.
5. EMS: EMS can be purchased from Sigma. EMS should be used in a fume hood. Wear gloves and a lab coat and avoid inhaling volatile substances.
6. Acetate buffer: Dilute glacial acetic acid to 100 m*M*, and pH to 5.0 with NaOH.
7. Sodium thiosulfate: Make fresh to 10% (w/v) in water. Filter-sterilize.
8. Canavanine medium: Canavanine stock is made to a final concentration of 30 mg/mL in water and filter-sterilized. Synthetic complete (SC) medium lacking arginine is composed of 0.67% (w/v) yeast nitrogen base (without amino acids), 2% (w/v) dextrose, 2% (w/v) bacto-agar, and any supplements required to compensate for genetic deficiencies in the yeast strain. Amino acids should be added from 100X stock solutions to a final concentration of 20 µg/mL for Arg, His, Met, and Trp; 30 µg/mL for Ile, Leu, Lys, and Tyr; 50 µg/mL for Phe; 100 µg/mL for Asp and Glu; 150 µg/mL for Val; 200 µg/mL for Thr; and 375 µg/mL for Ser. Bases are added to a final concentration of 20 µg/mL from 100X stock solutions. The medium is autoclaved at 15 psi for 15 min. After autoclaving, add canavavine to a final concentration of 30–40 µg/mL. Store at 4°C for up to 3 mo.
9. UV light source: Short wave (254 nm) UV light sources can be purchased. The UVGL-58 Mineralight from UVP (Upland, CA) is a handheld light source. Alternatively, a benchtop UV crosslinker can be used as a UV source.

3. Methods

3.1. EMS and MNNG Mutagenesis

1. Inoculate the yeast strain into 10 mL of YEPD broth. Incubate overnight at 30°C with shaking until the culture reaches a concentration of 2×10^8 cells/mL.
2. The next day, centrifuge 2.5 mL of the overnight culture in a screw-cap tube at 3000*g* for 4 min at 20°C. Wash the collected cells in 50 m*M* potassium phosphate buffer. Repeat with a second wash and resuspend in 10 mL of this buffer.
3. In a fume hood, add the optimal dose of MNNG or EMS to 10 mL of culture in a screw-cap tube. Mix culture well and incubate at 30°C for the previously determined time. For most laboratory strains, the optimal dose of MNNG will be between 4–10 µg/mL and EMS will have an optimal dose of about 3% of the final volume.
4. To stop MNNG and EMS mutagenesis, add an equal volume of 10% (w/v) filter-sterilized solution of sodium thiosulfate. Mix well (*see* **Note 1**).
5. Pellet the culture by centrifugation at 3000*g* for 4 min at 20°C. Pour off the supernatant and resuspend the cells in 10 mL of sterile water. Repeat centrifugation, pour off the supernatant, and resuspend in 1 mL of sterile water (*see* **Note 2**).
6. Plate cells on the appropriate medium to suit the experimental needs. Colonies usually appear after 2–4 d (*see* **Note 3**).
7. If mutation frequency is to be determined, cells should be plated after proper dilution onto both selective and nonselective media and resulting colonies must be counted.

3.2. UV Mutagenesis

1. Inoculate 10 mL of YEPD broth with host strain. Incubate the culture overnight at 30°C with shaking until the concentration reaches 2×10^8 cells/mL.
2. Pellet the culture by centrifugation at 3000*g* for 4 min at 20°C. Pour off the supernatant and resuspend the cells in sterile water. Repeat.
3. Spread 100 µL of an appropriate dilution of the cell suspension on each of several plates. Allow all liquid to be absorbed into the plate (*see* **Note 4**).
4. With lids removed, expose each plate to the optimal dose of UV. The optimal dose for most laboratory yeast strains is approx 50 J/m^2.
5. To avoid photoreactivation, incubate the plates in the dark for at least 24 h. Colonies usually appear after 2–4 d.
6. If mutation frequency is to be determined, cells should be plated after proper dilution onto both selective and nonselective media and resulting colonies must be counted.

4. Notes

1. The mutagenesis protocol can be adapted to suit the needs of any mutagen. Although some mutagens can be deactivated by addition of organic compounds, proper disposal of medium containing chemicals should be in accordance with local biosafety policies.

2. Enrichment procedures should be performed after the mutagen treatment and before plating. Cells should be transferred to rich medium and allowed to recover from the mutagenesis treatment. Enrichment procedures can be done on solid medium or a single mutant can be isolated from each of a series of liquid cultures to ensure independent origin of the mutants isolated.
3. By using large petri dishes (e.g., 150 mm diameter), one is able to plate up to 2000 colonies per plate, thus reducing the number of plates required for the screening.
4. Some UV light sources cast shadows at the edge of the petri dish. To avoid inaccurate results, do not spread the cells to the edges of the plate.

Acknowledgments

The authors wish to thank laboratory members for helpful discussion. This work is supported by the Canadian Institutes of Health Research operating grant MOP-38104 to WX.

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14

Gene Disruption in the Budding Yeast *Saccharomyces cerevisiae*

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Summary

One essential step for the molecular dissection of gene function is gene inactivation. In the yeast *Saccharomyces cerevisiae*, elaborate tools for gene disruption are available. Gene disruption cassettes carrying completely heterologous marker genes flanked by short DNA segments homologous to the corresponding regions left and right of the gene to be deleted result in highly efficient one-step gene disruption events yielding usually more than 50% of the clones carrying the correctly disrupted gene. Presence of *loxP* sites flanking the disruption marker gene allows Cre recombinase-mediated marker rescue so that the marker can be used to disrupt another gene.

Key Words: Single gene disruption; multiple gene disruptions; homologous recombination; sequence-specific recombination; heterologous marker genes; *loxP* site; Cre recombinase.

1. Introduction

Gene disruption is one of the most powerful techniques to study the function of a gene and of its product, the protein. Today, in the budding yeast *Saccharomyces cerevisiae*, the disruption of genes relies on the so-called one-step gene disruption approach, which is based on the fact that linear DNA fragments carrying a selectable marker gene with homology regions on either end to a yeast gene integrate at the corresponding chromosomal locus by homologous recombination with high efficiency ***(1,2)***. The fact that the flanking homology regions can be as short as 40–50 base pairs makes it possible to generate the gene disruption cassettes by polymerase chain reaction (PCR) thus omitting any time-consuming cloning steps. A scheme summarizing the individual steps of a PCR-mediated one-step gene disruption experiment is shown in **Fig. 1**. Today the favored selectable marker genes on the disruption cassette consist of

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

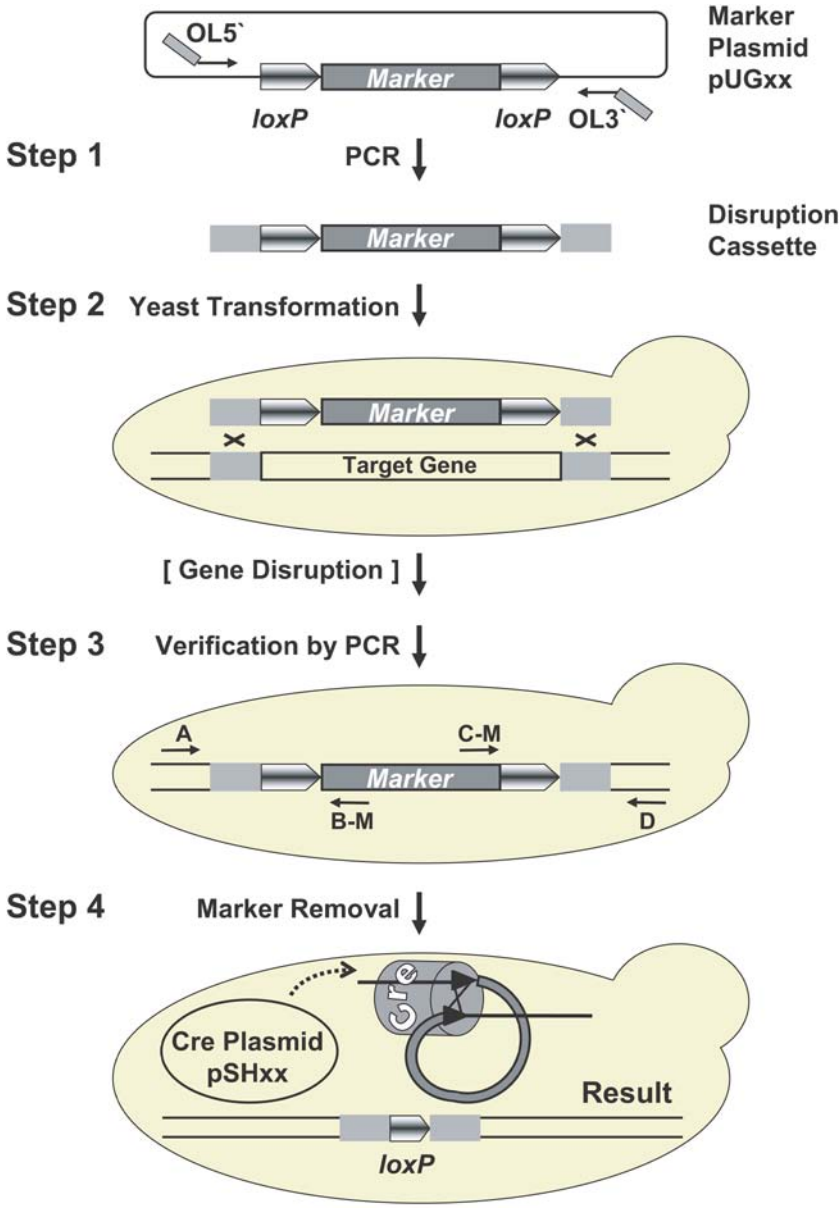


Fig. 1. General outline of the one-step gene disruption approach. A collection of marker plasmids (pUG series) carries the various selectable marker genes, each flanked by *loxP* sites that allow their subsequent removal from the genome. In Step 1, the disruption cassettes are generated by PCR using oligonucleotides that carry at their 3' ends sequences homologous to sequences left and right of the marker gene, and at their 5' ends sequences homologous to sequences that flank the target gene. After yeast transformation (Step 2), the disruption cassette integrates via homologous recombination

heterologous, completely non-*S. cerevisiae* DNA to maximize the chance of homologous recombination of the short flanking segments at both ends of the disruption cassette with their chromosomal counterparts. The first heterologous, dominant disruption marker was the *kan*^{r} gene-encoding resistance to Geneticin (G418), which has been used to generate a collection of more than 6000 disruption strains, each carrying a defined deletion of a particular yeast gene. This yeast gene knockout (YKO) collection is the primary source if a specific gene disruption strain is needed (*see* **Note 1**) ***(3,4)***. A detailed description of currently available disruption cassettes and their use can be found elsewhere ***(2)***.

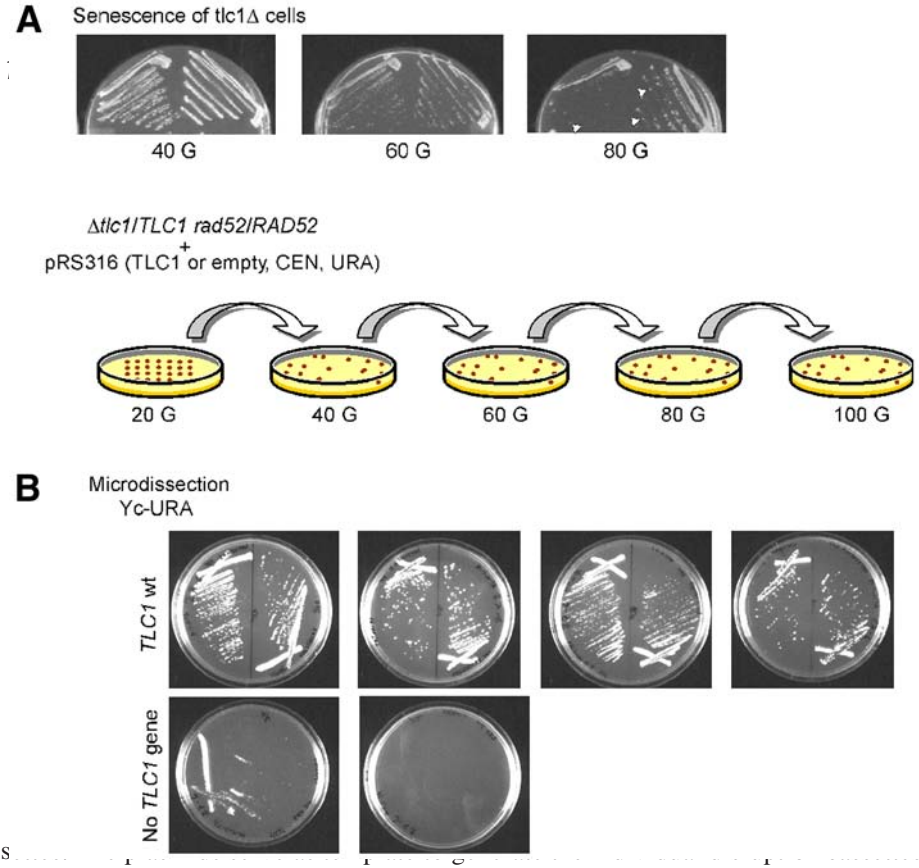
Often it is necessary to delete more than one gene, e.g., many cellular functions are maintained by several isoenzymes and thus their functional analysis requires disruption of more than one gene to uncover gene function. A well-known example in yeast is the hexose transporter family, where a concurrent knockout of at least 20 transporter genes was necessary to completely block growth on hexose sugars ***(5)***. Multiple gene disruptions can be done in two ways: (1) genes can be deleted sequentially using different gene disruption cassettes carrying different selectable markers; or (2) the disruption cassette can be removed from the genome by mitotic or recombinase-mediated recombination so that the disruption marker can be reused to disrupt another gene (overview in **ref. *2***). Removable disruption cassettes are the ones of choice, because they provide the greatest flexibility for later manipulations of the resultant strain. For the purpose of this protocol, we will focus on the use of a series of five completely heterologous *loxP*-flanked disruption cassettes, all of which can be efficiently removed by the Cre recombinase ***(6,7)*** (*see* **Note 2**). Other removable disruption cassettes rely on the action of the Flp recombinase or depend on a mitotic recombination event and have been summarized elsewhere ***(2)***.

2. Materials

2.1. Generation of Disruption Cassette

The pUG plasmid series carries gene disruption cassettes consisting of five completely heterologous marker genes (*kan*^{r}*, his5*$^+$*, ble, URA3, LEU2*) each flanked by *loxP* sites ***(7)*** (**Fig. 2**). Two cassettes carrying genes for resistance to the drugs geneticin/G418 (*kan*^{r}) and phleomycin (*ble*) that inhibit yeast

Fig. 1. *(continued)* into the genome replacing the target gene. PCR verification identifies yeast transformants harboring correctly integrated disruption cassettes (Step 3). If required, marker rescue is initiated by transforming a Cre expression vector into the disruptant strain, resulting in a strain in which the target gene is replaced by a single *loxP* site (Step 4).



All marker genes are from organisms other than *S. cerevisiae* and thus will not recombine with the *S. cerevisiae* genome. Expression of the marker genes is controlled by the *TEF2 Ashbya gossypii* promoter (P_{TEF}) and terminator (T_{TEF}), whereas the two *Klyveromyces lactis* genes are expressed from their own regulatory sequences (P and T, respectively). All five disruption cassettes can be generated by PCR using the same two primers OL5' and OL3'. The two primers comprise 19 or 22 3' nucleotides complementary to sequences in the pUG plasmids flanking the disruption cassettes and 45 5' nucleotides complementary to sequences upstream or downstream of the genomic target sequence to be deleted. The size of the disruption cassettes is indicated. The complete plasmid sequences can be found at GenBank under the following accession numbers: pUG6: AF298793; pUG27: AF298790; pUG66: AF298794; pUG72: AF298788; pUG73: AF298792 *(7)*. (*bla*, confers resistence against Ampicillin in *E. coli*; *ori*, origin of replication in *E. coli*; bp, base pairs; kbp, kilo base pairs; nt, nucleotides; P, promoter; T, terminator; *TEF*, translation elongation factor).

growth can be used to disrupt genes in any yeast strain (prototrophic industrial or wild-type strains).

2.1.1. Primer Design

All five disruption cassettes can be generated by PCR using the same oligonucleotides OL5' and OL3' (**Fig. 2**). The general design of OL5' and OL3' is as

follows: the 3' nucleotides of the primers are always 5' CAGCTGAAG CTTCGTACGC 3' (OL5'; upstream of the P_{TEF} resp. of the T elements) and 5' GCATAGGCCACTAGTGGATCTG 3' (OL3'; downstream of T_{TEF} resp. of the P elements). The sequences flanking the target gene in the genome are added to the 5' end of these sequences: 45 nucleotide stretches that are homologous to sequences upstream of the ATG start codon and downstream of the stop codon, respectively (**Fig. 1**). The 45 bp of flanking sequence on each side yields approx 80% correct integration of the disruption cassette (*see* **Note 3**). The primers to generate the disruption cassettes need to be of full-length, otherwise the chance of undesirable nonhomologous recombination increases (*see* **Note 4**). Care has to be taken that neighboring open reading frames (ORF) are not touched by the gene disruption event. Every deletion should be about 500 base pairs (bp) upstream of the next start codon and about 200 bp downstream of the next stop codon.

Many yeast genes and even chromosomal regions are duplicated in the genome. In these cases, it is necessary to check the 45 bp flanking homology sequences used for recombination to make sure that they are not present elsewhere in the genome. Moreover, several yeast genes are flanked by simple DNA sequences (e.g., poly[A/T] stretches downstream of a gene). Gene disruption cassettes carrying those stretches in the flanking homology sequences will give reduced numbers of transformants. In these cases, one can either find a completely new 45 bp homology sequence or create a longer flanking homology sequence by adding a unique sequence on either end.

2.1.2. Preparative PCR to Generate Disruption Cassette

1. Taq polymerase (various companies offer this enzyme; alternatively, the enzyme can be purified from a recombinant *Escherichia coli* clone ***(8)***.
2. 10X PCR buffer: 750 m*M* Tris-HCl, pH 9.0, 200 m*M* $(NH_4)_2SO_4$, 0.1% (w/v) Tween 20. Store at –20°C.

All chemicals should be of highest quality.

2.2. Yeast Transformation

Yeast transformation is done according to **ref. *9*** (*see also* Chapter 12).

1. Carrier DNA (2 mg/mL). High molecular-weight DNA (deoxyribonucleic acid Sodium Salt from Salmon Testes) (D1626, Sigma-Aldrich, Taufkirchen, Germany) is dissolved in sterile ddH_2O at 2 mg/mL. The DNA is dispersed into the solution by drawing it up and down repeatedly in a 10-mL pipet. The covered solution is mixed vigorously on a magnetic stirrer overnight in the cold room. Small aliquots of about 1 mL are stored at –20°C. Before use, the DNA has to be boiled at 100°C for 5 min and than chilled on ice.
2. 1 *M* lithium acetate stock solution (LiAc), pH 8.4–8.9. The solution is prepared in ddH_2O, filter-sterilized, and stored at room temperature.

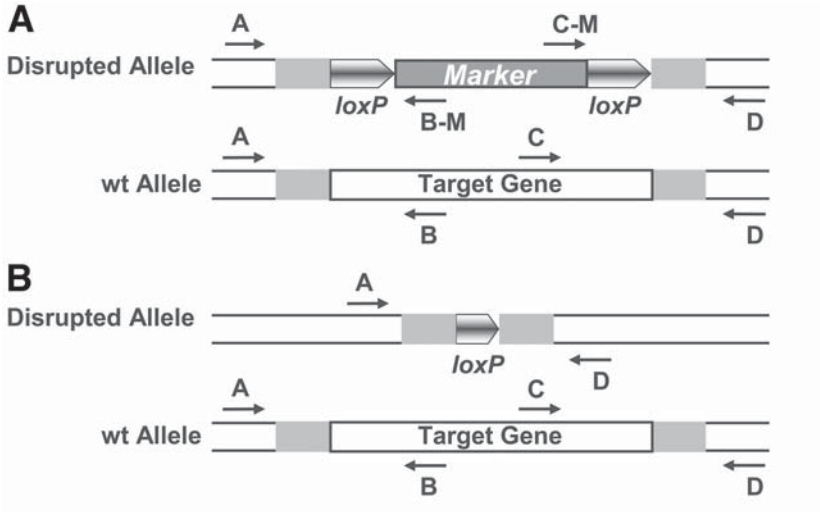
3. Polyethylene glycol (PEG 50% [w/v]). The PEG, MW 3350 (P3640, Sigma) is made up to 50% (w/v) with ddH_2O and filter-sterilized. It takes about 30 min to dissolve the PEG in the water. Store in aliquots of about 2 mL at –20°C. Avoid thawing and freezing. Just use twice or three times.
4. YPD medium (for details of yeast media, *see* **ref. *10***).
   a. 10 g yeast extract (e.g., 212750, BD, Heidelberg, Germany).
   b. 20 g peptone (e.g., 30392-021, Life Technologies, Paisley, Scotland).
   c. 14 g agar (for plates) (e.g., 214530, BD, Heidelberg, Germany).
   d. 2 mL adenine stock solution (2 mg/mL) in ddH_2O.
   e. 4 mL tryptophan stock solution (5 mg/mL) in ddH_2O.
   f. 20 g dextrose.

   Fill up with ddH_2O to 1 L and autoclave.
5. YPD + Geneticin. The active concentration of Geneticin (G418) may vary from lot to lot (500–800 µg/mg [w/w]); thus it is crucial that a final active concentration of 200 µg/mL is used (G418 plates can be tested by plating single cells of a G418-sensitive strain: no visible microcolonies should be formed). Add 200 mg of active Geneticin (e.g., 345810, Calbiochem, Merck KGaA, Darmstadt, Germany) dissolved in 1 mL sterile ddH_2O to 1 L of about 60°C warm YPD medium.
6. YPD + Phleomycin. Add 7.5 µg/mL Phleomycin (Phleo) (PHLEL0100, Cayla, Toulouse, France) to about 60°C warm medium.
7. SC-medium (for details of yeast media, *see* **ref. *10***).
   a. 20 g dextrose.
   b. 20 g agar (for plates).
   c. 1.7 g yeast nitrogen base (YNB) w/o amino acids and ammonium sulfate.
   d. 5 g ammonium sulfate.
   e. 2 g drop-out mix.

   Dissolve in 1 L ddH_2O and adjust the pH to approx 6.5 with 1 *M* NaOH.
8. Drop-out powder mix: The drop-out powder mix is the combination of the amino acids, bases, and chemicals listed in without the ones used for selection of the transformants. The powder mix needs to be vigorously mixed in a bottle by adding sterile marbles (Ø ~ 5mm). Shake to mix for at least 15 min (longer than you think necessary!).

   All chemicals should be of highest quality.

Adenine	2.0 g	Leucine	10.0 g
Alanine	2.0 g	Lysine	2.0 g
Arginine	2.0 g	Methionine	2.0 g
Asparagine	2.0 g	para-Aminobenzoic acid	0.2 g
Aspartic acid	2.0 g	Phenylalanine	2.0 g
Cysteine	2.0 g	Proline	2.0 g
Glutamine	2.0 g	Serine	2.0 g
Glutamic acid	2.0 g	Threonine	2.0 g
Glycine	2.0 g	Tryptophan	2.0 g
Histidine	2.0 g	Tyrosine	2.0 g
Inositol	2.0 g	Uracil	2.0 g
Isoleucine	2.0 g	Valine	2.0 g



C Disruption Cassette-specific Verification Primers 5' --> 3'

	B-M	C-M
Tn903 kan^r *S.p. his5$^+$* *Tn5 ble^r*	GGATGTATGGGCTAAATG	CCTCGACATCATCTGCCC
K.l. URA3	CAGACCGATCTTCTACCC	TTGGCAAATCATGACCCC
K.l. LEU2	AGTTATCCTTGGATTTGG	ATCTCATGGATGATATCC

Fig. 3. PCR-based verification of a gene disruption in a diploid yeast strain. **(A)** To confirm the correct integration of the disruption cassette, PCR reactions are performed using combinations of the corresponding target gene-specific (primers A, B, C, D) and disruption cassette-specific primers (B-M and C-M). PCR products of the expected size will be obtained only if the integration of the disruption cassette was successful. **(B)** Cre-mediated removal of the marker can be verified by PCR using the primers A and D. **(C)** The DNA sequences of the general disruption cassette-specific primers B-M and C-M.

2.3. Verification of Correct Clone/Gene Disruption by PCR

2.3.1. Primer Design

To check if the transformants have integrated the disruption cassette correctly, PCR analysis of yeast transformants is performed (**Fig. 3A**). The PCR primers A to D flanking the disrupted gene should be chosen such that the PCR products generated (PCR products of primers A, B, C, D and disruption cassette-specific primers B-M and C-M, as shown in **Fig. 3A,B**) are between 500

bp and 1000 bp in length. Therefore, the A oligonucleotide should bind about 300 bp upstream of the integration cassette in the genome, while the D oligonucleotide should bind about 300 bp downstream of the disruption cassette. Oligonucleotides B and C amplifying the transitions from the endogenous gene to the surrounding genomic area should bind within the target gene about 300 bp away from the start and stop codon. The primers should have melting temperatures of 63–67°C. The disruption cassette-specific primers are listed in **Fig. 3C**.

2.3.2. PCR Verification

The necessary reagents are the same as those listed before (*see* **Subheading 2.1.2.**)

2.4. Marker Rescue/Repeated Gene Disruption

The pSH plasmid series carries the *cre* gene under regulation of the galactose-inducible *GAL1* promoter *(7)* (**Fig. 4**).

1. YPG medium. This is the same medium as YPD but 2% galactose is used as the carbon source instead of glucose.

3. Methods

To disrupt a gene, one has to transform yeast cells with a linear DNA fragment carrying a marker gene that provides a selectable phenotype (usually prototrophy or resistance to drugs), flanked by sequences homologous to sequences flanking the gene to be deleted (**Fig. 1**). A set of different selectable marker genes is available that encodes resistance to drugs or prototrophy for amino acids or nucleotide bases. These completely heterologous marker genes are all flanked by two *loxP* sites that allow Cre-mediated recombination resulting in efficient marker rescue *(7)* (**Fig. 2**) (*see* **Note 2**). The disruption cassette is generated via PCR using oligonucleotides with their 3' 19–22 nucleotides homologous to sequences flanking the disruption marker on a plasmid and their 5' 45 nucleotides homologous to sequences left and right of the gene to be deleted (**Fig. 1**, step 1). Next, the disruption cassette is transformed into yeast cells using a high-efficiency tranformation protocol (**Fig. 1**, step 2). The disruption cassette integrates into the genome by homologous recombination, thus precisely replacing the target gene. To confirm correct integration of the cassette into the genome, yeast transformants are analyzed by PCR using combinations of the corresponding target gene-specific and disruption cassette-specific primers (**Fig. 1**, step 3). PCR products of the expected size will be obtained only if the disruption cassette has integrated correctly. Finally if a disruption marker needs to be removed from the genome, a Cre expression

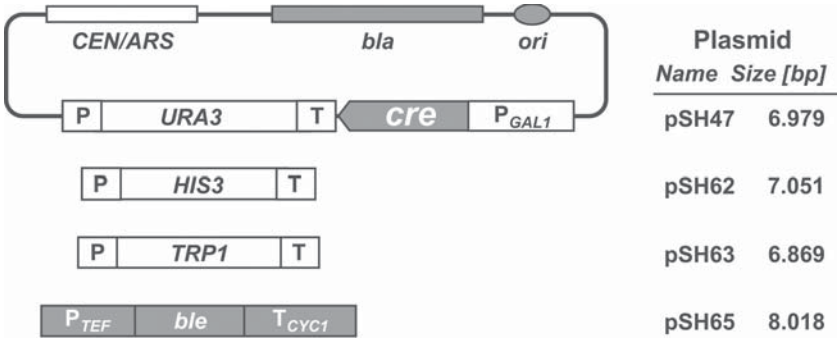


Fig. 4. The collection of Cre-expressing pSH plasmids *(7)*. Cre expression is regulated by the galactose-inducible *GAL1* promoter. Shifting yeast cells transformed with these plasmids to galactose media results in expression of Cre, followed by Cre-induced recombination of the *loxP* sites flanking the disruption marker gene, leaving behind a single loxP site at the original site of disruption cassette integration. Different selection markers maximize the use of the Cre system. The complete plasmid sequences can be found at GenBank under the following accession numbers: pSH47: AF298782; pSH62: AF298785; pSH63: AF298789; pSH65: AF298780. *bla*, confers resistence against Ampicillin in *E. coli*; *ori*, origin of replication in *E. coli*; bp, base pairs; P, promoter; T, terminator; *TEF*, translation elongation factor; *CYC1*, Cytochrome c.

plasmid is transformed into the disruptant strain. Induction of Cre expression induces a *loxP*-mediated recombination event, resulting in loss of the marker gene, leaving behind a single *loxP* site at the site of the deleted target gene (**Fig. 1**, step 4).

3.1. Generation of Disruption Cassette

The disruption cassettes are generated by preparative PCR. As as template, one of the plasmids described in **Fig. 2** will be used. Set up the PCR reaction as follows:

1. 100 pmol OL5' (50 pmol/µL)	2 µL
2. 100 pmol OL3' (50 pmol/µL)	2 µL
3. 200 µ*M* dNTPs (4 m*M*)	5 µL
4. 1.5 m*M* $MgCl_2$ (25 m*M*)	6 µL
5. 10X buffer	10 µL
6. Template DNA (~50 ng)	1 µL
7. Taq polymerase (~0.5 U)	1 µL
8. ddH_2O	73 µL
	100 µL

PCR conditions:

Initial step (Hot start)	5 min	95°C	} 25 cycles
Denaturation	40 s	94°C	
Annealing	1 min	58°C	
Extension	2 min	68°C	
Final extension	15 min	68°C	

Each PCR should yield about 500 µg of PCR product. For each transformation, combine the product of two PCR reactions. DNA-precipitate the PCR product and resuspend in 34 µL sterile ddH_2O (*see* **Note 5**).

3.2. Yeast Transformation (according to ref. 9*) (*see *Note 6)*

1. Inoculate a yeast strain into 5 mL YPD medium and incubate overnight on a rotary shaker at 30°C.
2. Determine the titer of the yeast culture by counting. Count budded cells as one cell. Some strains form clumps of cells. Therefore vigorously vortex cells prior to counting.
3. Transfer 2.5×10^8 cells to 50 mL fresh YPD-medium to give 5×10^6 cells/mL.
4. Incubate the flask on a shaker at 30°C. It is important to allow the cells to complete at least two divisions. This will take 3–5 h. The transformation efficiency (transformants/µg plasmid/10^8 cells) remains constant for three to four cell divisions.
5. When the cell titer is at least 2×10^7 cells/mL, harvest the cells by centrifugation at 1600*g* for 5 min, wash the cells in 25 mL of sterile ddH_2O, and resuspend in 1 mL 0.1 *M* LiAc. Transfer the cell suspension to a 1.5-mL microfuge tube and centrifuge for 10 s at top speed (10,000–13,000*g*) at room temperature and discard the supernatant.
6. Boil the carrier DNA as described before (*see* **Subheading 2.2.**).
7. Resuspend the cells in 0.5 mL 0.1 *M* LiAc to maintain a cell titer of 2×10^9 cells/mL.
8. For each transformation reaction pipet 50 µL samples into 1.5-mL microfuge tubes, centrifuge at top speed for 10 s and remove the supernatant.
9. Add the following in the given order:
   240 µL PEG
   36 µL 1 *M* LiAc
   50 µL boiled carrier DNA
   34 µL DNA plus water (500–1000 ng of the disruption cassette)
   Total: 360 µL
10. Vortex each tube vigorously until the cell pellet is been completely resuspended.
11. Incubate the cells for 30 min at 30°C.
12. Incubate the cells for 30–40 min at 42°C. (The optimal time may vary for different yeast strains.)
13. Centrifuge at top speed for 10 s and remove the supernatant with a micropipet.
14. In case of selection for a prototrophy, resuspend the pellet in 200 µL sterile ddH_2O and spread onto two selective plates, 100 µL per plate.

15. In case of selection for a resistance, resuspend the cells in 1 mL YPD and incubate for at least 1 h on a rotator at 30°C.
16. Centrifuge at top speed for 10 s and remove the supernatant.
17. Resuspend the pellet in 200 µL sterile ddH_2O and spread onto two selective plates, 100 µL per plate.
18. Incubate plates 3 to 5 d at 30°C. Expect between 10 and 100 tranformants on each plate.
19. G418 plates have to be replica plated after 24–36 h onto fresh G418 plates.

3.3. Verification of Correct Clone/Gene Disruption by PCR

To confirm that the disruption cassette is integrated correctly in the genome and has replaced the gene you wanted to disrupt, you have to prepare different PCR reactions as outlined in **Fig. 3A,B**. Using the primer combinations A/B-M and C-M/D, you only get a specific PCR product if the deletion cassette has integrated at the correct place (**Fig. 3A**).

In about 8% of the gene disruption events, a gene deletion is accompanied by a duplication of the gene (duplication of the entire chromosome or of a particular chromosomal region). Therefore the absence of the deleted gene needs to be tested by PCR using primer combinations A/B and C/D (**Fig. 3A**). A PCR with oligonucleotides A and D amplifying the entire locus gives you a further hint for a correct disruption. Here, care has to be taken in cases where the A/D PCR fragments obtained from the disrupted allele and from the wt allele are of similar size. The A/D PCR may not be easy to achieve depending on the size of the DNA fragment you need to amplify. On average, between 50% and 80% of the transformants will be correct by PCR criteria.

In **Fig. 5**, an example of a successful gene disruption experiment is presented. A *YNL107w*/*YAF9*-specific *kanMX* disruption cassette was transformed into a haploid yeast strain and transformants checked by verification PCR.

Set up the PCR reaction as follows:

1. 25 pmol primer 1 (50 pmol/µL)	0.5 µL
2. 25 pmol primer 2 (50 pmol/µL)	0.5 µL
3. 200 µ*M* dNTPs (4 m*M*)	1.25 µL
4. 1.5 m*M* $MgCl_2$ (25 m*M*)	1.5 µL
5. 10X buffer	2.5 µL
6. Taq polymerase (~0.25 U)	0.5 µL
7. Yeast cells	
8. ddH_2O	18.25 µL
	25 µL

Colony purify the yeast transformants on selective plates and then on a YPD plate (add wild-type strain as negative control). For the PCR, use only freshly grown yeast cells (no more than 2 d old) and never refrigerated. To add cells to

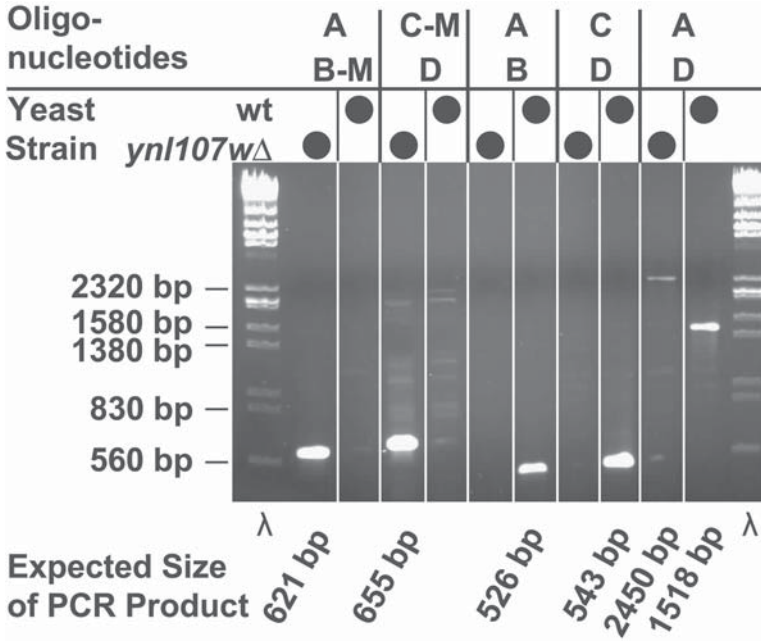


Fig. 5. Example of a successful gene disruption experiment in a haploid yeast strain. PCR analysis to confirm correct integration of the *kanMX* gene disruption cassette at the *YNL107w* / *YAF9* locus. The disruption cassette was generated by PCR using plasmid pUG6 as template and *YNL107w*/*YAF9*-specific oligonucleotides OL 5' and OL 3' (for sequences, *see* **Note 8**). Colony-purified yeast transformants were controlled for correct integration of the disruption cassette by PCR using target gene-specific primers A to D (for sequences, *see* **Note 8**) and the *kan*^{r}-specific B-M and C-M primers (for sequences, *see* **Fig. 3C**). The size of the expected PCR products is given below each lane. A successful gene disruption in a haploid yeast strain is characterized by the absence of PCR products for the primer combinations A-B and C-D (*see* **Fig. 3A**). wt, nontransformed wild-type yeast strain; *ynl107wΔ*, yeast strain carrying the correctly disrupted gene *YNL107w*; (λ = *Hin*dIII/*Eco*RI digested λ-DNA).

the PCR reaction, just touch the surface of a yeast colony with a yellow pipet tip so that you can just barely see the cells on the end of the pipet tip. Resuspend these cells in the PCR mix (*see* **Note 7**). Too many cells or agarose will inhibit the PCR reaction!

PCR conditions:

Initial Step	5 min	94°C	
Denaturation	1 min 30 s	94°C	} 35 cycles
Annealing	2 min	50°C	
Extension	2 min 30 s	72°C	
Final extension	7 min	72°C	

Depending on the oligonucleotides you have designed for the verification, you may have to adjust the annealing temperature.

3.3.1. Important: Occurrence of Collateral Mutations

Each yeast transformation randomly generates mutations in the genome. In gene disruption experiments, between 5 and 10% of the transformants carry a second-site (or collateral) mutation resulting in a growth phenotype ***(2)***. To avoid this problem, one should work with diploid strains homozygous for the disruption. The diploid strain should be generated by crossing two independently generated haploid disruption strains of the opposite mating type. This way most collateral mutations are complemented (most of them are recessive). If one needs to work with hapoids disruption strains, it would be best to cross the originally generated haploid disruption strain back several times to the corresponding wild-type strain.

3.4. Marker Rescue/Repeated Gene Disruption

To disrupt a second gene in a yeast strain, one can either use a disruption cassette with a different genetic marker or the first gene disruption cassette can be removed from the genome so that the marker can be used a second time. In the case of the *loxP* flanked disruption cassettes, the Cre expression plasmid needs to be transformed into the strain. Induction of Cre expression by growing transformants in galactose-containing media is followed by identification of yeast cells that have lost the disruption cassette marker. Loss of the marker can be easily verified by appropriate PCR reactions as outlined in **Fig. 3B**. Subsequently the Cre plasmid is removed from this yeast strain, which is now ready for a second disruption experiment.

1. Transformation of the desired Cre expression plasmid (**Fig. 4**) as described before.
2. Selection for transformants by plating out on selective media. Colony purify single transformants.
3. Incubate single colonies in 5 mL YPG medium overnight.
4. Plate about 100–200 cells onto YPD plates and incubate them for 1 d at 30°C.
5. Replica plate onto two plates: (1) selective for the marker on the disruption cassette and (2) on YPD. Alternatively, about 12 colonies can be streaked out onto a selective and a YPD plate. Cells that cannot grow on the selective medium have lost the disruption cassette. Pick cells from the corresponding colonies/streak out from the YPD plates. More than 50% of the colonies will have lost the marker of the disruption cassette.
6. To verify marker loss, perform the appropriate PCR reactions as shown schematically in **Fig. 3B** (*see* **Subheading 3.3.**).
7. To remove the Cre expression plasmid from a marker-minus yeast strain incubate cells in 5 mL YPD medium overnight. The next morning, shift 200 µL of the cells to 5 mL fresh YPD medium. In the evening, shift 50 µL of the cells to 5 mL fresh YPD medium. Always incubate the cells at 30°C on a rotator.

8. Plate 100–200 cells onto YPD plates and incubate for 1 d at 30°C.
9. Replica plate onto two plates: (1) selective for the Cre-expressing plasmid and (2) on YPD. Alternatively, about 12 colonies can be streaked out onto a selective and a YPD plate. Cells that cannot grow on the selective medium have lost the *cre* plasmid (Expect between 5 and 50% of the colonies to be positive). Corresponding colonies on the YPD plates can be streaked out on fresh YPD plates (*see* **Note 9**).
10. Finally test again for loss of disruption cassette marker and Cre plasmid marker by streaking cells onto selective plates.

4. Notes

1. The entire YKO collection or single gene disruption strains thereof can be obtained from the following companies:
   American Type Culture Collection (ATCC), P.O. Box 1549, Manassas, VA 20108, USA. Phone: (703) 365-2700. E-mail: news@atcc.org. http://www.biospace.com/company_profile.cfm?CompanyID=69904
   EUROSCARF, Institute for Microbiology, Johann Wolfgang Goethe-University Frankfurt, Marie-Curie-Strasse 9; Building N250, D-60439 Frankfurt, Germany. FAX: +49-69-79829527. E-mail: Euroscarf@em.uni-frankfurt.de. http://www.rz.uni-frankfurt.de/FB/fb16/mikro/euroscarf/
   Invitrogen GmbH, Technologiepark Karlsruhe, Emmy-Noether Strasse 10, 76131 Karlsruhe, Germany. Phone: +49-800-0830902. FAX: +49-800-0833435. E-mail: euroinfo@invitrogen.com. http://www.invitrogen.com
   Invitrogen Corporation, 1600 Faraday Avenue, P.O. Box 6482, Carlsbad, CA 92008, USA. Phone: (760) 603-7200. FAX: (760) 602-6500. http://clones.invitrogen.com/cloneinfo.php?clone=yeast
   Open Biosystems, 6705 Odyssey Drive, Huntsville, AL 35806, USA. Phone: (888) 412-2225 or (256) 704-4848. Fax: (256) 704-4849. E-mail: info@openbiosystems.com. http://www.openbiosystems.com/yeast_knock_outs.php
2. The cloned disruption cassettes (pUG plasmid series) as well as the various Cre expression plasmids (pSH plasmid series) are available from EUROSCARF (Frankfurt, Germany) (*see* **Note 1** for complete address) or from our lab.
3. In rare cases, it can be hard to obtain correct transformants using the usual 45 bp of flanking homology, probably because homologous recombination is impeded (e.g., by a particular chromatin structure). Usually a gene disruption cassette flanked by 90–100 bp of homology solves this problem.
4. Make sure that oligonucleotides used to create the disruption cassette are full-length. 5' shortened oligonucleotides will reduce the efficiency of homologous recombination. To check the quality of oligonucleotides, one can load 2 µL of a 50 pmol/µL solution onto a 3–4% agarose gel. Comparison with control oligonucleotides of defined length gives a rough quality check.
5. It is not necessary to separate the PCR product from the template plasmid DNA, because all the disruption cassette-carrying pUG plasmids cannot be inherited by yeast cells. This has to be checked if other cloned disruption cassettes are used as

template. If the plasmid used as template in the PCR to generate the disruption cassette is able to replicate autonomously in yeast cells (because it contains an ARS sequence), then many yeast transformants will carry the plasmid rather than the disruption cassette.

6. Details of the yeast transformation protocol can be found at: (http://www.umanitoba.ca/faculties/medicine/biochem/gietz/Solutions.htmL)
7. Alternatively, you can boil the amount of about 5 µL yeast cells in 50 µL 0.02 *M* NaOH for 15 min at 100°C and add 1 µL of this solution to the PCR mix. Genomic yeast DNA can also be prepared according to Chapter 2 and an aliquot can be added to the PCR reaction.
8. Oligonucleotides used for the disruption and verification of *YNL107w* (5'→3'). Lower case letters indicate nucleotides homologous to sequences left and right of the cloned disruption cassettes (*see* **Fig. 2**).
   OL5' ACTTGTGACCACCTATTTACGGCATCACAAAGAAAGCGAGcagctgaagcttcgtacgc
   OL3' TGGCTGTTATGAAAATACCGTTGTTCCGGGTGCAGTGATCgcataggccactagtggatctg
   A GTTCAACACCGTGTTCCG
   B CATAAATGAGTATGTTCG
   C GACAGAATAGAGATCGGC
   D AAATTCAGGTGTGTCCAC
9. The *GAL1* promoter expressing the Cre recombinase is already weakly active in glucose media. If you are in a hurry, you can also incubate the cells for 2 d in YPD medium and then streak out and replica plate onto selective and YPD plates. About 1 to 5% of the colonies will have lost the marker of the disruption cassette.

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15

Inducible Degron and Its Application to Creating Conditional Mutants

R. Jürgen Dohmen

Summary

Conditional mutants are important tools particularly in the analysis of essential genes. In this chapter, a method is described that allows for a rapid design-based generation of temperature-sensitive alleles of many *Saccharomyces cerevisiae* genes. The method employs a temperature-inducible degron, denoted as *td*, which, when linked to the N-terminus of proteins to be studied, targets them for rapid degradation via the ubiquitin-dependent N-end rule pathway. Targeting, however, occurs only at elevated (restrictive) temperatures, whereas at lower (permissive) temperatures the degron is inactive. Strategies to generate *td* alleles are described, and the limitations of the method are discussed.

Key Words: Conditional mutations; degron; degradation; ubiquitin; N-end rule.

1. Introduction

Conditional mutants are invaluable tools in the characterization of gene function. These mutants can be cultivated at the permissive condition under which a mutant allele functions more or less as its wild-type counterpart. Upon shifting to the nonpermissive condition, the mutant protein rapidly loses its function. The consequences of this loss can then be studied using the full repertoire of genetic, cell biological, and biochemical methods, many of which are described in this volume.

In the classic procedure, strains with point mutations obtained by random mutagenesis are selected that confer, e.g., heat or cold sensitivity to a given gene function. This is an often tedious and time-consuming strategy that does not always yield the desired conditional alleles. Another problem is that the alleles obtained are often too leaky to give clear results in functional assays. A more design-based procedure is the utilization of conditional promoters that allow for phenotypic or biochemical analyses after promoter shutoff. The dis-

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

advantage of the latter method is that, depending on the half-life of the mRNA and of the protein, it may take many hours until the protein of interest is eliminated. The slow decline in protein level might be accompanied by cellular adaptation such as induction of stress responses and the gradual appearance of cellular defects, including ones that are indirect consequences of the primary defects.

To circumvent these problems, several methods have been developed that are based on a conditional degradation of the protein of interest. These methods are based on fusions, in which the protein of interest is linked to a sequence that functions as a degradation signal, or "degron." Various methods have employed the so-called "N-degron," which targets proteins carrying it for degradation by the ubiquitin(Ub)-mediated N-end rule pathway.

The N-end rule relates the stability of proteins to the nature of their N-terminal residues. Proteins carrying N-terminally destabilizing residues (N-degron, below denoted as "N-deg") such as arginine (R) or leucine (L) are recognized by the Ubr1 Ub ligase (**Fig. 1**) ***(1,2)***. Ubr1 forms a complex with the Ub conjugating enzyme Ubc2/Rad6. This complex mediates the attachment of a Lys-48 linked poly-Ub chain to substrate proteins bearing an N-degron, provided they contain suitable internal Lys residues that can serve as attachment sites for ubiquitylation ***(3–6)***. Polyubiquitylated substrates are subsequently recognized and degraded by the proteasome ***(7,8)***. The Ubr1 protein has independent binding sites for two types of N-terminal residues. One binding site recognizes basic (type I) amino acids (Arg, Lys, and His); the other site binds bulky hydrophobic (type II) residues (Phe, Leu, Trp, Tyr, and Ile) ***(2)***. In addition, Ubr1 bears a third binding site for internal less well-characterized degrons ***(9)***. Among the functions of the N-end rule pathway are the regulation of peptide uptake and the degradation of cohesin fragments derived from separin-mediated cleavage ***(9,10)***. Despite these functions, mutants with inactivated N-end rule pathway enzymes such as *ubr1-* are viable and grow at rates similar to wild type ***(5,10)***. Null *ubr1* mutants therefore are suitable to control the effects of the modifications that go along with the generation of *td* alleles (*see* **Fig. 2**) in the absence of proteolytic targeting.

Because those residues that target proteins for degradation via the N-end rule pathway do not occur naturally as the result of translation, the ubiquitin (Ub) fusion technique can be employed to generate fusions that expose the desired residue at the resulting N-terminus after cotranslational processing by Ub-processing proteases ***(1,11)***. These proteases cleave precisely after the C-terminal Gly-76 residue of Ub irrespective of the following amino acid sequence ***(12–14)***. As illustrated in **Fig. 2**, a fusion protein Ub-N-deg-Poi upon its synthesis will instantly be processed to yield N-deg-Poi (protein of interest carrying an N-degron). What are the strategies to make the degradation of such

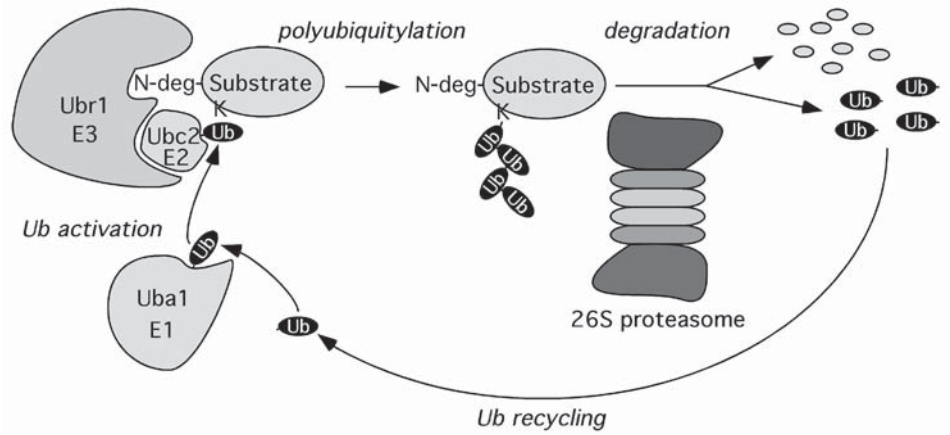

Fig. 1. Enzymes of the N-end rule pathway. The C terminus of ubiquitin (Ub) is activated by Ub-activating enzyme (Uba1 or E1). In this ATP-dependent process, Ub is covalently bound to a Cys residue of E1 via a thioester bond. Ub is then transferred to the Ub-conjugating enzyme (E2) Ubc2/Rad6. Ubc2 forms a complex with the Ub protein ligase (E3) Ubr1, also known as N-recognin. The latter binds to the N-degron of its substrate proteins and together with Ubc2 mediates the attachment of a polyUb chain to a Lys (K) residue of the substrate. The polyUb chain is recognized by subunits of the 19 S activator subcomplex of the 26 S proteasome, which degrades polyubiquitylated proteins down to small peptides. Ub is released from the substrate and thus recycled by proteasome-associated Ub isopeptidase activity.

a protein conditional? One possibility is to drive expression of a protein that is destabilized as described previously from a conditional promoter. High expression of an N-degron-tagged version of *ARD1* from the strong galactose-inducible P_{GAL1} promoter, for example, has been shown to serve as a growth-permissive condition. Shutting of expression by the addition of glucose resulted in rapid disappearance of the protein ***(15)***. In a related "two-pronged" approach, a tightly regulated version of P_{CUP1} was used simultaneously to shut off expression of the gene of interest and to induce expression of *UBR1*. The former was achieved by the P_{CUP1}-mediated induction of a repressor that specifically repressed the promoter driving the gene of interest ***(16)***. Similar to this approach is the utilization of P_{GAL1} promoter-driven expression of *UBR1* to achieve an inducible degradation of an N-degron tagged protein (R. J. Dohmen, K. Madura, B. Bartel, and A. Varshavsky, unpublished results). All these strategies require the change of media and are accompanied by artificial expression levels of the genes of interests under the permissive conditions.

The strategy described here that uses a temperature-inducible N-degron (*td*) does not require any change of media, and can in principle be achieved with

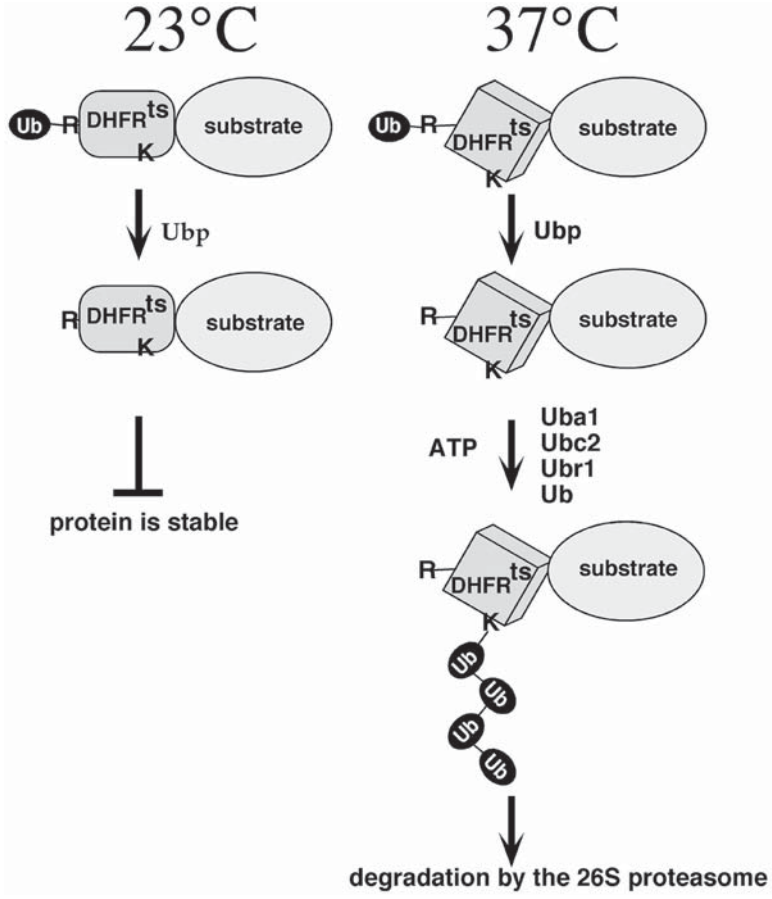

Fig. 2. Principle of inducible protein knockdown mediated by the temperature-inducible degron (*td*). The protein of interest is expressed as a fusion protein bearing N-terminal ubiquitin (Ub) followed by a residue that is destabilizing according to the N-end rule and a temperature-sensitive version of mouse dihydrofolate reductase (DHFR). The N-terminal Ub moiety is cotranslationally cleaved by Ub-processing proteases (Ubps), yielding a fusion protein with arginine (R) as the N-terminal residue. At the permissive temperature, this fusion protein is stable because the N-terminal R is not sufficiently exposed and/or because no suitable lysine (K) residue is available for ubiquitylation. As a consequence, the degron-bearing protein is stable at this temperature. The nonpermissive temperature induces conformational changes or a local unfolding that exposes one or both of the aforementioned elements of the N-degron. As a consequence, the protein is ubiquitylated by the Ubr1/Ubc2 complex and thereby targeted for degradation by the proteasome.

expression from the authentic promoter ***(17)***. A *td* variant of a protein of interest is generated by fusing a modified and temperature-sensitive mouse dihydrofolate reductase (DHFR) moiety to its N terminus. The modified DHFR is expressed as a Ub fusion (Ub-R-DHFR) to expose N-terminal Arg (R-DHFR) after cleavage by Ub processing proteases (**Fig. 2**). It was found that R-DHFR is a stable protein, despite the fact that Arg is a destabilizing residue according to the N-end rule ***(3,17)***. One interpretation of this result was that R-DHFR does not bear the second determinant of an N-degron, a Lys (K) residue that is sufficiently exposed to be ubiquitylated by the Ubr1/Ubc2 complex (**Fig. 2**) ***(3,17)***. A mutant version, R-DHFR^{td}, was isolated, the degradation of which by the N-end rule pathway was heat-inducible (***17***). The exchange of a Pro to a Leu residue in position 66 of R-DHFR^{ts} apparently results in a conformational change that provides sufficient accessibility to an internal Lys residue for its ubiquitylation. Experiments that involved extension of the N-terminus of R-DHFR^{ts}, however, indicated that in addition an increased exposure of the N-terminal Arg residue is likely to be the main reason for the heat inducibility of the N-degron of this protein (**Fig. 2**) ***(18)***. The mutated R-DHFR^{ts} was demonstrated to constitute a transplantable temperature-inducible degron (*td*), which allows for an easy in vivo knockdown of proteins carrying it by simply shifting the cells to a higher temperature ***(17)***. Since the first description of this strategy, numerous studies have successfully employed *td* alleles of essential genes (e.g., ***19–25***). More recently, a modified polymerase chain reaction (PCR)-based procedure has been applied to systematically generate *td* alleles of essential genes in *Saccharomyces cerevisiae* ***(26)***. The *td* strategy has also been used to generate conditional *Schizosaccharomyces pombe* mutants ***(27)***.

Among the limitations of the N-end rule-based *td* procedure is that it can neither be applied to proteins that do not tolerate an N-terminal extension nor to proteins present in compartments, such as those of the secretory pathway that are not accessible to the N-end rule machinery. The principle of the *td* strategy, however, could also be applied to such proteins if other degrons that are recognized in other degradation pathways are employed (*see*, e.g., ***28***; *see* **Note 1**).

2. Materials

2.1. Common Materials

1. Yeast synthetic minimal medium with 2% Dextrose (SD): 6.7 g/L yeast nitrogen base without amino acids, 2% glucose.
2. Yeast strain carrying auxotrophic marker mutations such as *ura3, leu2, trp1,* or *his3*, e.g., JD47-13C, and an isogenic diploid strain such as JD51 ***(29)***.

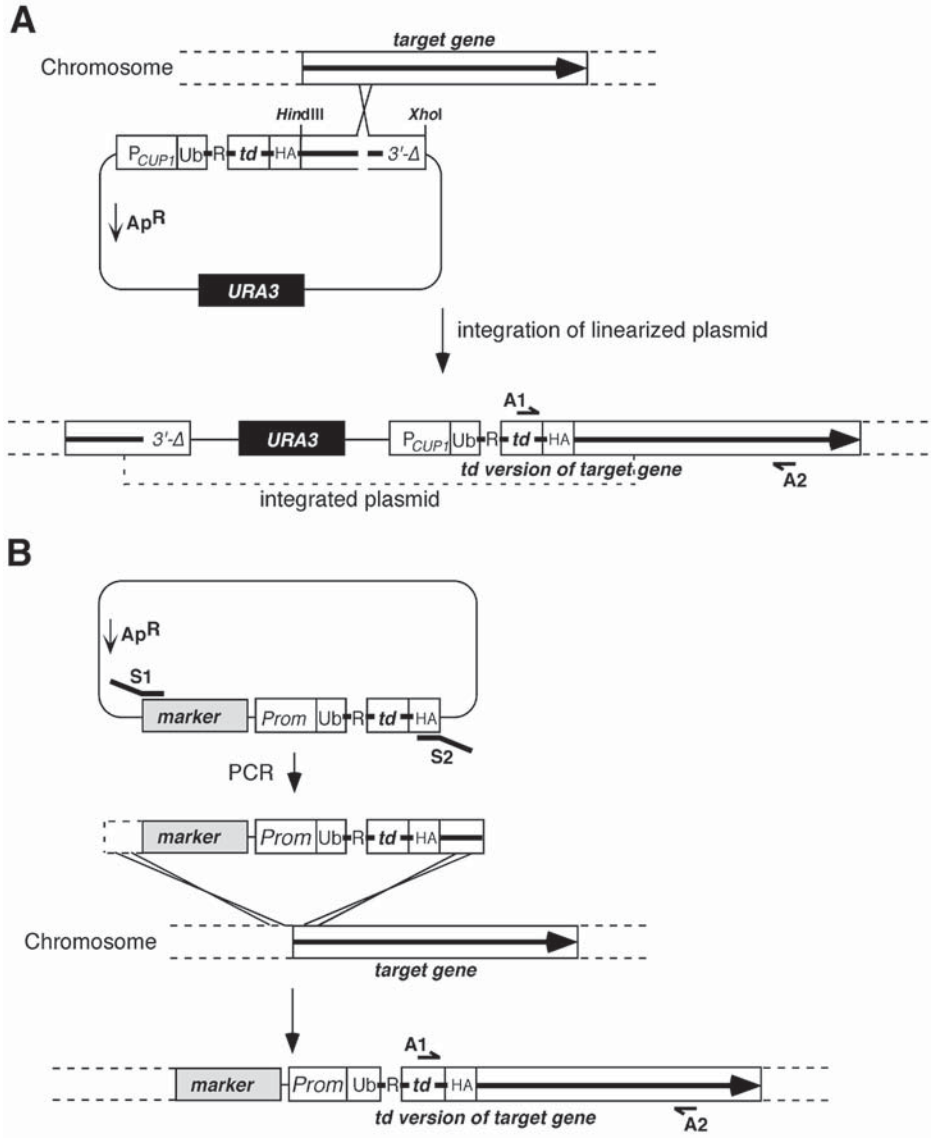

Fig. 3. Strategies for the generation of *td* alleles. **(A)** *Td* tagging via plasmid integration. First, an integrative plasmid is constructed, in which a 5' portion of the target gene that is usually generated by PCR is inserted in frame with a Ub-R-DHFR^{ts} (Ub-R-*td*) sequence located downstream of a promoter (here P_{CUP1}). For targeted integration, the plasmid is linearized with a restriction endonuclease within the sequence of the target gene. Integration via homologous recombination at the genomic target locus results in a 3' truncated inactive version of the target gene followed by its active *td* version. Yeast transformation with the correctly integrated plasmid can be identified by an analytical PCR that uses primers A1 and A2, which are specific, respectively,

3. Yeast strain carrying the *UBR1* gene under the strong galactose-inducible P_{GAL1} promoter, e.g., JD54 ***(29)***.
4. Isogenic control yeast strain that carries a *ubr1-* mutation, e.g., JD55 ***(29)***.
5. Integrative vector with a selectable marker such as *URA3, LEU2, TRP1,* or *HIS3* ***(30,31)*** that contains a promoter driving the expression of the Ub-DHFR^{td} cassette (**Fig. 3**). In the strategy outlined in **Subheading 3.1.1.**, pPW66R, a *URA3*-based integrative plasmid derived from pRS316 ***(31)***, which was used to produce a *cdc28*^{td} strain ***(17)***, is a starting point for the construction of *td* alleles of other genes.
6. Yeast DNA as a template for PCR amplification of gene fragments.
7. PCR reagents, including a high-fidelity thermostable polymerase mix, which can be purchased from various suppliers.

2.2. Yeast Transformation

1. Solution A: 1 *M* sorbitol, 3% ethylene glycol, 5% dimethyl sulfoxide (DMSO), 10 m*M* bicine-NaOH, pH 8.35.
2. Solution B: 40% polyethylene glycol (PEG) 1000 (Roth, Karlsruhe, Germany), 0.2 *M* bicine-NaOH, pH 8.35.
3. Solution C: 0.15 *M* NaCl, bicine-NaOH, pH 8.35.
4. 10 mg/mL calf thymus DNA (Sigma, Taufkirchen, Germany); heat-denature for 5 min in a boiling water bath and cool down rapidly on ice.

Fig. 3. *(continued)* for the DHFR sequence and the target gene outside of the sequence that was present in the integrative plasmid. In the approach shown, the *td* allele is driven by the copper-inducible P_{CUP1}. As an alternative, the authentic promoter of the target gene can be used instead. This approach requires an additional cloning step: the insertion of a PCR product containing the promoter. The latter strategy (not shown) has the advantage that, after plasmid integration, a pop-out of sequence resulting from recombination between the repeated promoter sequences results in a stable *td* allele of the target gene expressed from its natural promoter at the authentic genomic location. **(B)** Single-step, PCR-based "short flanking homology" strategy of *td* tagging. A plasmid containing the *td* tag expressed from a promoter (Prom) such as P_{GAL1} or P_{CUP1} preceded by a selectable marker gene is used as a template in a PCR reaction. As a result of this PCR that uses primers S1 and S2, short sequences (~45 nt) homologous to the target gene are attached to the marked *td* module. The forward primer S1 matches the 3' sequence of the target gene's promoter, whereas the reverse primer S2 matches that of the Crick strand of the 5' end of the target gene ORF. The PCR product is directly used to transform yeast cells. Homologous recombination between the short flanking sequences of the PCR product and the target gene result in a stable insertion of the marked *td* tagging module between the target gene's promoter and ORF. Whether correct targeting has occurred within the selected transformants can be verified quickly using the same analytical PCR assay as described in **(A)**.

3. Methods

3.1. Generation of td Mutants

In principle, three strategies for the generation of *td* mutants can be envisioned. The first one, which will not be described here in detail, is to introduce a *td* allele of the gene of interest on a plasmid into a strain carrying a deletion of the respective gene. If the gene is essential, this can be achieved either by selecting spore clones containing both the plasmid and the deletion allele, or by a plasmid shuffle strategy. In the latter case, a strain whose deletion is covered by a plasmid carrying the wild-type allele is transformed with another plasmid containing the *td* allele. Subsequently, clones that have lost the plasmid with the wild-type allele during mitotic divisions are selected.

The second strategy is based on the integration of a plasmid carrying a 3' truncated version of the *td* allele at the genomic locus of the gene of interest (**Fig. 3A**) ***(17)***. Integration of the plasmid via homologous recombination will result in a genomic locus expressing the *td* allele as well as a truncated nonfunctional version of the gene (*see* **Note 2**).

The third strategy (**Fig. 3B**) introduces the *td* allele stably into the genome via homologous recombination of a cassette carrying the *td* tag downstream of a promoter and a selectable marker gene ***(22,32)***.

3.1.1. Construction of Integrative Plasmids for the Generation of td Alleles

1. About 0.5–1.0 kb of the 5' end of the open reading frame (ORF) of the gene of interest are amplified by PCR using a high-fidelity polymerase with two specifically designed primers. These should have 20–25 nucleotides (nt) identical to their target sequences and they should carry appropriate restriction sites at their 5' ends for cloning. When pPW66R (***17***) is used, the 5' oligonucleotide should introduce a *Hin*dIII site upstream of the ATG start codon. We used an oligonucleotide that introduces two Gly codons downstream of the *Hin*dIII site in front of the ATG start condon (CGCCAA GCT TCC GGG GGG ATG...). The Gly residues were intended to serve as a flexible hinge between the *td* domain and the protein of interest. The 3' oligonucleotide should introduce an *Xho*I site. If possible, the fragment should contain a single restriction site that is absent from plasmid pPW66R (*see* **Note 3**).
2. Cleave PCR fragment and vector pPW66R with *Hin*dIII and *Xho*I.
3. Isolate large fragment of pPW66R and cleaved PCR product from an agarose gel.
4. Ligate isolated fragments.
5. Transform *Escherichia coli* cells with ligation products and select ampicillin-resistant colonies.
6. Prepare plasmid DNA from *E. coli* transformants and verify the desired restriction pattern.
7. Linearize plasmid with a restriction endonuclease that cleaves once in the plasmid within the target sequence, because this results in greatly increased efficiency of targeted integration ***(33)*** (*see* **Note 3**).

3.1.2. Generation of td *Alleles by Genomic Transplacement*

An alternative strategy uses PCR products with so-called "short flanking sequences" to produce *td* alleles by genomic transplacement ***(22,32)***. In this procedure, a plasmid such as pFA6a-kanMX6-tsDegron-3HA, pFA6a-TRP1-tsDegron-3HA, or pFA6a-His3MX6-tsDegron-3HA, in which a P_{GAL1} driven Ub-DHFR^{ts} is preceded by a kanamycin-resistance marker conferring resistance to G418, a *TRP1* marker or a *HIS3* marker, respectively ***(32)***, or plasmid pKL187, in which the *td* tag is controlled by P_{CUP1} and preceded by the kanamycin-resistance marker ***(22)*** is used as a template in a PCR using primers S1 and S2 (**Fig. 3**). The forward primer S1 bears approx 45 nt identical to the 3' end of the target gene's promoter followed by approx 20 nt identical in sequence to the region flanking the marker gene within the *td* cassette. The reverse primer S2 is made of approx 45 nt of the Crick strand of the 5' end of the target gene ORF followed by approx 20 nt identical to the 3' end of the *td* tag. The 45 nt short flanking sequences are usually sufficient to mediate transplacement of genomic sequence by in vivo recombination on yeast transformation ***(34–37)*** (*see* **Note 4**).

1. Design target gene-specific primers S1 and S2 as outlined above and illustrated in **Fig. 3** (*see* **Note 4**).
2. Amplify the *td* tagging cassette by PCR using a high-fidelity polymerase and primers S1 and S2.
3. The PCR product (1–2 µg) can directly be used for yeast transformation.

3.1.3. Transformation of Frozen Competent Yeast Cells

For the generation of *td* alleles, an easy and rapid transformation protocol is recommended that allows the freezing of aliquots of competent cells ***(38)***. The transformation rates, although lower than those that can be obtained by the protocol described by D. Gietz within this volume, are more than sufficient to obtain a large number of the desired transformants. Because usually the same wild-type strains, and strains lacking or inducibly overexpressing *UBR1,* are used for the generation of various *td* mutants, multiple aliquots of the competent cells can be prepared and stored at –80°C until a plasmid or PCR product is ready for transformation (*see* **Note 5**).

1. Grow culture of a wild-type yeast strain such as JD47-13C, an isogenic diploid strain such as JD51 and an isogenic *ubr1-* mutant such as JD55 (*ubr1-*), as well as a strain that overexpresses *UBR1* such as JD54 (P_{GAL1}-*UBR1*) in YPD (10 mL per transformation) to an optical density (OD) measured at 600 nm of 0.6–1.0 (*see* **Note 6**).
2. Spin down cells at 1200*g* for 5 min.
3. Resuspend the pellet in 0.5 volumes (5 mL per transformation) of solution A.
4. Spin down cells at 1200*g* for 5 min.

5. Resuspend cells in 0.02 volumes (200 µL per transformation) of solution A.
6. Freeze 200 µL aliquots of the competent cells in microfuge tubes at –80°C (*see* **Note 7**).
7. For transformation, take an aliquot of the frozen competent cells and add the linearized plasmids described in **Subheading 3.1.1.** or the PCR product described in **Subheading 3.1.2.**, and 50 µg calf thymus DNA. Thaw cells at 37°C (room temperature also works) with rapid agitation for 5 min.
8. Add 1.2 mL of solution B and mix gently.
9. Incubate 60 min at 30°C without shaking.
10. Spin down cells at 1200*g* for 5 min.
11. Wash cells with 1.2 mL solution C.
12. After spinning down cells again, decant supernatant, resuspend cells in the remaining supernatant, and plate on selective media.
13. Select transformants by incubation at 25°C for 4–5 d.

3.1.4. Verification of Recombinant Clones by Analytical PCR

Both strategies described above (**Fig. 3**) yield yeast transformants bearing modified versions of the target genes at their authentic genomic location. In both cases, however, those transformants that result from correct genomic targeting need to be identified among those resulting from undesired recombination events. This is most rapidly achieved by analytical PCR using one primer (A1) specific for the ts degron, and one primer (A2) that hybridizes to the target gene outside of the sequences present in the DNA used for transformation (**Fig. 3**). Primer A could be an approx 25 nts oligonucleotide with a sequence identical to the 3' end of the DHFR ORF.

1. Design and acquire primers A1 and A2.
2. Tranfer a small amount of cells from a selected yeast transformant into a PCR tube using a sterile 200-µL pipet tip.
3. Heat denature cells in the microwave for 1 min.
4. Set up analytical PCR (up to 35 cycles) using primers A1 and A2. Use untransformed yeast cells as a negative control.
5. Analyze PCR products by agarose gel electrophoresis. Clones giving rise to the expected PCR product should carry the *td* allele in the genome (*see* **Note 8**).

3.2. Phenotypic and Biochemical Analysis of td *Mutants*

The first step in the phenotypic analysis of the transformants carrying the *td* allele is to test their growth at the temperature that leads to the induction of the ts degron (usually 37°C). If a *td* allele was produced of an essential gene, the correct transformants are therefore expected to be unable to grow at 37°C. If no tight ts phenotype can be observed, transformants of a strain overexpressing *UBR1* (such as JD54) can be tested also, because this leads to a more rapid

degradation of N-end rule substrates ***(22,39)***. As a control, otherwise identical transformants generated in a *ubr1-* background (e.g., strain JD55) can be used to assay the phenotype in the absence of Ubr1-mediated degradation.

The turnover of the *td* tagged protein can be monitored by Western blot analysis of extracts obtained from cells after various times of incubation at 37°C. HA epitopes present in the construct (*see* **Fig. 3**) enable specific and sensitive detection of the protein of interest with anti-HA antibodies available from various suppliers.

If the *td* clones display a tight ts phenotype and a rapid turnover of the *td* tagged protein, the constructed mutants are suitable for assays that study the role of the protein under investigation in a given process.

4. Notes

1. If your protein of interest does not tolerate or is functionally impaired by an N-terminal extension, the *td* approach is obviously not suitable to generate conditional versions of it. One alternative strategy would employ a degron that can be attached to the C terminus of the protein ***(28)***. Another approach is to conditionally target the ubiquitylation machinery to the protein of interest via transplanted protein interaction domains. Methods have been described, in which a domain known to interact with the protein of interest was fused to ubiquitin-conjugating enzymes (Ubc) or to an F-box subunit of the SCF-Ring finger ubiquitin ligase ***(40,41)***.
2. The truncated nonfunctional 5' fragment of the gene that goes along with the integrative strategy (*see* **Fig. 3A**) should only encompass a few hundred nt in order to minimize the risk that an inhibitory protein fragment is generated. Such an effect is excluded either by a two-step protocol as described in the caption to **Fig. 3A**, or by the PCR-based approach shown in **Fig. 3B**.
3. If it is impossible to find a restriction enzyme that linearizes the integrative plasmid by producing a single cut within the target sequence, look for enzymes that cut twice in the plasmid and perform a partial digestion. This still produces sufficient clones with correctly integrated plasmid.
4. Details on the design of primers S1 and S2 containing short flanking sequence can be found in the papers describing the respective template plasmids ***(22,32)***. Occasionally, the short flanking strategy does not yield correct recombinants. In those cases, another strategy can be used, in which longer flanking sequences are produced by an assembly PCR protocol ***(32,42)***.
5. Parameters critically affecting the transformation efficiency with this procedure, are the source of the PEG 1000, and efficient mixing of the samples during and after thawing.
6. An isogenic strain set such as JD47-13C, JD51, JD55, and JD54 is recommended for the analysis. The diploid strain is important if no correct viable transformants are obtained with the wild-type haploid. This can either be explained by ineffi-

cient genomic targeting, or by lethality of the N-terminal extension of the protein under investigation. In the latter case, correct targeting could only be detected in the diploid strain. A tetrad analysis would further confirm that the construct is lethal. In such cases, the *td* strategy is not suitable for the gene studied (*see* **Note 1**). If for various reasons, another strain background is preferred, an isogenic set of haploid, diploid, and *ubr1-* strains should be used. *Ubr1-* constructs for the generation of the latter marked with *LEU2* or *HIS3* are available upon request. Overexpression of *UBR1* can either be achieved by placing the genomic copy under the control of a strong promoter, as is the case in strain JD54 ***(29)***, or by introducing plasmids enabling strong ectopic expression of *UBR1*. Such plasmids are available upon request.

7. Cells can either be frozen by directly placing them into the –80°C freezer or by dipping them into dry ice/acetone. Freezing in liquid nitrogen reduces transformation efficiency significantly. Frozen competent cells can be stored in the freezer for a long period until they are needed.
8. The analytical PCR shown in **Fig. 3** only confirms the correct targeting of sequences to the desired locus in the genome. There is, however, the possibility that positive transformants, in addition, contain a wild-type allele of the gene of interest. Such a constellation may result from cell fusion, which occurs occasionally upon transformation. The resulting transformant, although mating as a haploid, would be a diploid strain that is heterozygous with respect to the *td* mutation. In such cases, a second analytical PCR can be performed to determine the presence or absence of a wild-type genomic copy of the locus under investigation. Primer A2 (**Fig. 3**) can be used together with a primer that anneals to the promoter of the gene studied. This PCR, however, is likely not to yield any product in the correct clones because, owing to the insertion of the *td* cassette, it would be several kb in size. The absence of a PCR product, however, is usually not completely satisfactory as a proof for a desired genotype. As an alternative, the genomic rearrangements and the absence of a wild-type copy of the gene can be confirmed by Southern blot analysis. Such additional measures will usually not be required if the results of the analytical PCR correlate with the occurance of the expected ts phenotype. Complementation of the ts phenotype with a wild-type copy of the gene will then be sufficient to verify the genotype.

Acknowledgments

Research in the author's laboratory is funded by the DFG (Do 649). I wish to express my gratitude to Alexander Varshavsky, on whose idea the *td* strategy is based and in whose laboratory it was developed together with Pei-Pei Wu.

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16

Synthetic Lethal Screen

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Summary

The synthetic lethal screen is a method of isolating novel mutants whose survival is dependent on a gene of interest. Combining the colony-color assay with a synthetic lethal screen offers a means to visually detect a mutant that depends on a plasmid for survival. Screening for synthetic lethals can be achieved in four steps. First, the gene of interest must be mutated in a strain harboring the *ade2 ade3*/*ade8* mutations and producing white colonies. A plasmid containing the *ADE3*/*ADE8* gene and the wild-type gene of interest must then be transformed into the strain, which results in red colonies with white sectors where the plasmid has been lost. A mutagenesis is then required to introduce random mutations into the yeast genome. Any cell with a mutation that causes dependence on the gene of interest for survival must maintain the plasmid; these cells will produce solid red colonies. Finally, the mutants are transformed with a library. The mutants containing complementing DNA are no longer dependent on the plasmid carrying the gene of interest and thus the synthetic lethals are identified by their red-white sectoring phenotype. The synthetic lethal gene can be identified by isolating and sequencing plasmid DNA.

Key Words: Yeast; genetic screen; mutant; synthetic lethal; method.

1. Introduction

The lower eukaryote *Saccharomyces cerevisiae* has been used as a model organism to study gene function. It is possible to systematically analyze lethality and other phenotypes resulting from deletion of each gene ***(1)***. The synthetic lethal screen is a powerful genetic screen that relies on finding secondary molecular targets. In principle, a synthetic lethal screen can identify any gene that, if mutated, causes cell death with a nonlethal "primary" mutation. Through the use of synthetic lethal screening, the entire genome of an organism can be scanned to identify mutations in related pathways or proteins with redundant functions.

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

The synthetic lethal screen is a method of isolating novel mutants whose survival is dependent on a gene of interest. A synthetic lethal screen works on the premise that a desired mutant is reliant on a plasmid containing the gene of interest to survive and form colonies. Current synthetic lethal screening protocols use the colony-color assay that was developed independently by both Koshland et al. ***(2)*** and Hieter et al. ***(3)*** in 1985. This assay relies on the ability to visually identify plasmid loss in a single colony by using the *ade2* and *ade3*/*ade8* mutations. The *ADE2* gene is required in the purine biosynthesis to convert P-ribosylaminoimidazole (AIR) to P-ribosylaminoimidazolecarboxylate (CAIR). Strains that harbor the *ade2* mutation accumulate the intermediate AIR, which results in accumulation of a red pigment. *ADE3*/*ADE8* is involved in the metabolism of tetrahydrofolate (THF); three enzymes, methyleneTHF dehydrogenase, methenylTHF cyclohydrolase, and formylTHF synthetase are encoded by the *ADE3*/*ADE8* locus ***(4)***. This mutation also blocks a branch of the histidine pathway, making the *ade3*/*ade8* strain a histidine auxotroph ***(5)***. The *ade3*/*ade8* mutation is epistatic to *ade2* and blocks the pathway, at a point prior to pigment accumulation, resulting in an *ade2 ade3*/*ade8* double mutant that forms white colonies. Introducing a plasmid carrying *ADE3*/*ADE8* into an *ade2 ade3*/*ade8* strain generates red colonies containing white sectors where the plasmid has been lost ***(2,3)***. Combining the colony-color assay with a synthetic lethal screen offers a means to visually detect a mutant that depends on a plasmid for survival ***(6,7)***.

The efficiency of the synthetic lethal screen is strongly influenced by the stability of the plasmid carrying the gene of interest. YRp-based plasmids are extremely unstable and complete plasmid loss occurs within a few generations ***(8)***. YCp plasmids are the vector of choice for synthetic lethal screens; however, the high level of stability of centromere plasmids generates a high number of false positives that must be further characterized. The existing synthetic lethal screen protocol can by improved by regulating the plasmid stability and copy number. It was found that by placing a yeast centromere sequence under the control of an inducible promoter, plasmid stability could be significantly decreased under inducing conditions. By altering the conditions under which the strain carrying the plasmid P_{GAL1}-*CEN4* is cultured, one is able to develop a method that eliminates virtually 100% of false-positives and thus reduces the time required to carry out a synthetic lethal screen ***(9)***.

Screening for synthetic lethals can be achieved in four steps. First, the gene of interest must be mutated in a strain harboring the *ade2 ade3*/*ade8* mutations and producing white colonies. A plasmid containing the *ADE3*/*ADE8* gene and the wild-type gene of interest must then be transformed into the strain. The colonies produced will show a distinct red phenotype with white sectors where the plasmid has been lost. A mutagenesis is then required to introduce random

Table 1
Expected Color Phenotype for Each Step of the Synthetic Lethal Screen

Genotype	Phenotype[a]
1. *ade2 ade3* Strain will carry deletion in gene of interest.	White
2. *ade2 ade3*/YCpU-ADE3 Plasmid will carry wild-type copy of gene of interest.	Red-white sectors
3. *ade2 ade3 gene X* / YCpU-ADE3 Mutagenesis will introduce mutation in gene X. Plasmid is required for survival.	Red
4. *ade2 ade3 gene X* / YCpU-ADE3 pGENE X LEU2 Library plasmid carrying *GENE X*.	Red-white sectors

[a]Phenotype is indicated for plating on rich medium.

mutations into the yeast genome. Finally, select for any cell with a mutation that causes dependence on the plasmid for survival. These cells can readily be identified by screening for solid red colonies (*see* **Table 1**).

In order to determine the synthetic lethal genes, the mutants are transformed with a library. The mutants containing complementing DNA are no longer dependent on the plasmid carrying the gene of interest and thus the synthetic lethals are identified by their red-white sectoring phenotype. The synthetic lethal gene can be identified by isolating and sequencing plasmid DNA ***(6,7)***.

If using standard auxotrophic markers for selection, at least three markers will be needed in the yeast strain. Although the *ade3* mutation renders the strain a histidine auxotroph, the *HIS3* marker can be used to disrupt the original gene if used before the *ade3* mutation is incorporated into the strain. If using this combination, one must consider future experiments that will depend on selection of the *HIS3* marker in this strain. This may become important in backcrossing the mutation with an isogenic strain of the opposite mating type. If available, the *URA3* marker should be used for the original transforming plasmid. Selection on 5-fluoroorotic acid medium allows for a powerful selection against uracil prototrophs ***(10)***. Having this selection available will be useful in testing for the dependence on the plasmid for survival after the mutagenesis assay and also in conjunction with the library screen to allow for a positive selection.

The following protocol has been adapted from ***(9)*** and is based on the use of a centromeric plasmid under the control of a *GAL1* promoter (*see* **Fig. 1**).

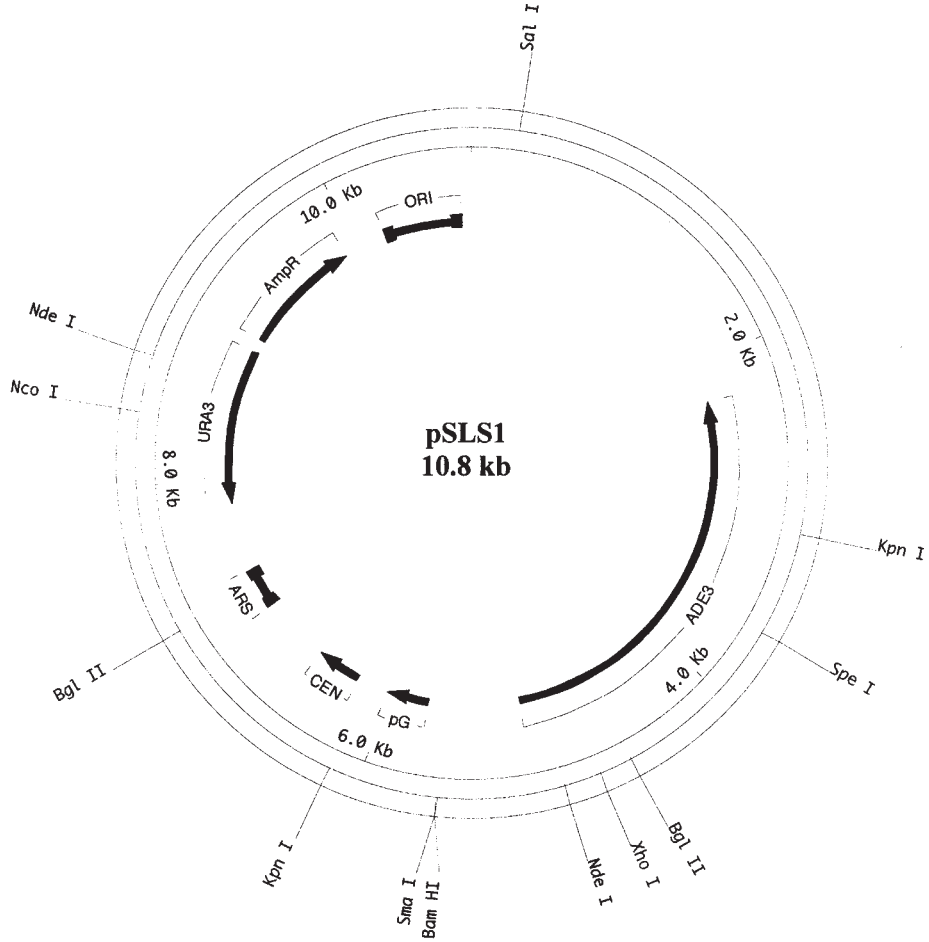

Fig. 1. Schematic diagram of plasmid pSLS1 used for the synthetic lethal screen. The plasmid contains markers for bacteria (Amp^R), and yeast (*URA3*) selection, and autonomous replicators (*ORI* and *ARS*, respectively). It also contains a wild-type copy of *ADE3* and a centromere sequence (*CEN4*) under the control of an inducible *GAL1* promoter (pG). Your favorite gene can be cloned into the plasmid using a unique cloning site such as *Bam*HI, *Sal*I, or *Sma*I.

2. Materials

1. Water for solutions and media should be distilled and deionized (ddH_2O).
2. YEPD medium: 1% (w/v) yeast extract, 2% (w/v) bacto-peptone, and 2% (w/v) dextrose, dissolved in water, and autoclaved at 15 psi for 15 min. Liquid medium can be solidified using 2% (w/v) bacto-agar. Store liquid medium at room temperature and solidified medium at 4°C for up to 3 mo.

3. Auxotrophic marker agar plates: Synthetic dextrose (SD) medium is composed of 0.67% (w/v) yeast nitrogen base (without amino acids), 2% (w/v) dextrose, 2% (w/v) bacto-agar, and any supplements required to compensate for genetic deficiencies in the host strain except those to be used as a selectable marker. Amino acids should be added from 100X stock solutions to a final concentration of 20 µg/mL for Arg, His, Met, and Trp; 30 µg/mL for Ile, Leu, Lys, and Tyr; 50 µg/mL for Phe; 100 µg/mL for Asp and Glu; 150 µg/mL for Val; 200 µg/mL for Thr; and 375 µg/mL for Ser. Bases are added to a final concentration of 20 mg/mL from 100X stock solutions. The medium is autoclaved at 15 psi for 15 min and can be stored at 4°C for up to 3 mo.
4. Transformation TE: 10 m*M* Tris-HCl and 0.1 m*M* ethylenediaminetetraacetic acid (EDTA) at pH 7.6 (sterilized through autoclaving).
5. Li-TE: 0.1 *M* lithium acetate in transformation TE (sterilized through autoclaving).
6. PEG: 50% (w/v) solution of polyethylene glycol (PEG) 4000 in water (sterilized through filtration).
7. 10 µg/mL stock salmon sperm DNA (ssDNA) (Sigma, St. Louis, MO): dissolve in ddH_2O, shear by repeated passage through an 18-gage needle, aliquot, and store at –20°C. Before use, boil for 5 min and immediately chill on ice.
8. MNNG or EMS: Both *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) and ethyl methanesulfonate (EMS) can be purchased from Sigma. The MNNG solution should be made in a fume hood with the window lowered as much as possible. Gloves and a lab coat should be worn and inhalation of MNNG powder should be avoided. Dispense 10 mg of MNNG into a capped, pre-weighed glass vial. Re-weigh and add a sufficient volume of acetate buffer to bring the concentration to 1 mg/mL. MNNG should be used immediately or dispensed into Eppendorf tubes for storage at –20°C. Each stock tube of MNNG should only be used once and thawed on ice immediately before use. MNNG is light-sensitive and should be stored in the dark. EMS should be used in a fume hood. Wear gloves and a lab coat and avoid inhaling volatile substances.
9. Acetate buffer: Dilute glacial acetic acid to 100 m*M* and adjust pH to 5.0 with NaOH.
10. Sodium thiosulfate: Make fresh to 10% (w/v) in water. Filter-sterilize.
11. 50 m*M* potassium phosphate buffer: Make at pH 7.0.

3. Methods

3.1. Preparation of Host Strain

Disruption of the gene of interest should be done in a host strain that contains *ade2* and *ade3* deletions (*see* **Note 1**). The gene disruption should be confirmed phenotypically and genotypically. A wild-type copy of this gene will then be transformed back into the strain on a single-copy plasmid containing *ADE3* (P_{GAL1}-*CEN4*). A standard transformation protocol can be used to accomplish both steps. The following protocol has been adapted from Ito et al. ***(11)***.

1. Grow a fresh overnight culture of the recipient host strain in YEPD broth at 30°C with shaking.
2. The next day, use 1 mL of this culture to inoculate 9 mL of YEPD broth.
3. Grow cells to a density of 1×10^7 cells/mL at 30°C with shaking.
4. Transfer 1.5 mL of the culture to a microfuge tube. Pellet the cells by spinning at 16,000*g* for 15 s. Pour off the supernatant.
5. Wash the cells once in 400 µL Li-TE and resuspend in 100 µL Li-TE.
6. Add 4 µL of boiled ssDNA (from 10 µg/mL stock) and 4 µL (approx 1 µg) of plasmid DNA to the microfuge tube followed by 280 µL of PEG. Mix the contents by inverting the tube several times.
7. Incubate at 30°C for 45 min.
8. Heat-shock cells by placing at 42°C for 5 min.
9. Pellet the cells at 16,000*g* for 15 s. Pour off the supernatant, wash cells with sterile water.
10. Resuspend the cells in 100 µL sterile water and plate directly onto selective agar. Incubate at 30°C for 2–4 d.

After setting up the aforementioned strain, ensure the color phenotype is correct and the plasmid can be lost at a high rate on galactose medium (*see* **Note 2**).

3.2. Mutagenesis

The mutagenesis experiment can be carried out using any of several different mutagens; MNNG and EMS are used most frequently. Mutagens should be used in a fume hood and with appropriate protective clothing. A standard mutagenesis protocol can be used with minor variations (*see* **Note 3**). Selection of the plasmid should be maintained in the overnight culture to ensure high efficiency in recovery of synthetic lethal mutants. The plating medium should contain galactose as the carbon source. Dilute and plate for growth of all viable cells.

3.2.1. EMS and MNNG Mutagenesis

1. Inoculate the yeast strain in 10 mL of YEPD broth. Incubate overnight at 30°C with shaking until the culture reaches a concentration of 2×10^8 cells/mL.
2. The next day, centrifuge 2.5 mL of the overnight culture by centrifuging in a screw-cap tube at 3000*g* for 4 min at 20°C. Wash the collected cells in 50 m*M* potassium phosphate buffer. Repeat with a second wash and resuspend in 10 mL of this buffer.
3. In a fume hood, add the optimal dose (*see* **Note 3**) of MNNG or EMS to 10 mL of culture in a screw-cap tube. Mix culture well and incubate at 30°C for the previously determined time. For most wild-type laboratory strains, the optimal dose of MNNG will be between 4 and 10 mg/mL and EMS will have an optimal dose of 3% of the final volume.

4. To stop MNNG and EMS mutagenesis, add an equal volume of 10% (w/v) filter-sterilized solution of sodium thiosulfate. Mix well.
5. Pellet the culture by centrifugation at 3000 *g* for 4 min at 20°C. Pour off the supernatant and resuspend the cells in sterile water. Repeat.
6. Resuspend the cells in sterile water and plate on the appropriate medium to suit the particular experimental needs. Colonies usually appear after 2–4 d.

3.2.2. UV Mutagenesis

1. Inoculate host strain in 10 mL of YEPD broth. Incubate overnight at 30°C with shaking until a concentration of 2×10^8 cells/mL is reached.
2. Pellet the culture by centrifugation at 3000*g* for 4 min at 20°C. Pour off the supernatant and resuspend the cells in sterile water. Repeat.
3. Resuspend the cells in sterile water and spread 100 µL of an appropriate dilution of the cell suspension on each of several plates. Allow all liquid to be absorbed into the plate (*see* **Note 4**).
4. With lids removed, expose each plate to the optimal dose of ultraviolet (UV) light (*see* **Note 3**). The optimal dose for most wild-type laboratory yeast strains is approx 50 J/m^2.
5. To avoid photoreactivation, incubate the plates in the dark for at least 24 h. Colonies usually appear after 2–4 d.

3.3. Selection of Synthetic Lethal Mutants

Cells that contain a mutation that is synthetic lethal in conjunction with the deletion of the gene of interest will appear as a solid red colony on the galactose medium. Cells that do not contain synthetic lethal mutations will appear as red and white sectoring colonies (*see* **Note 5**). Because the cells are plated on a galactose medium, the plasmid will become unstable and will be easily lost in subsequent generations. Once putative synthetic lethal mutants have been recovered, several rounds of testing should be completed before carrying out a library screen (*see* **Note 6**). To determine whether a synthetic lethal mutation is one of those already known, wild-type copies of each known synthetic lethal gene can be transformed into the mutant strain. Plasmids that complement the second mutation will allow cells to lose the original plasmid and sectoring colonies will appear on galactose medium, thus eliminating the need to screen with a library.

3.4. Library Screen

The library should be chosen based on the needs of the library screen. Single-copy or multi-copy library plasmids can be used. A small-scale library screen should be performed to determine transformation efficiency before carrying out a large-scale screen. A standard transformation protocol can be scaled up to suit the needs of the experiment.

Library transformants can be directly selected by growing the cells on selective minimal medium with galactose (*see* **Note 2**). Library plasmids containing complementary DNA will allow the loss of the original plasmid resulting in a red and white sectoring colony. To ensure that the complementing phenotype is dependent on the plasmid and not owing to other events, the mutant should be screened for the ability to lose the library plasmid and return to the original phenotype. This can be accomplished by isolating the library plasmid and transforming back into the mutant strain carrying the original plasmid. Selection on FOA plates or on galactose medium should determine the ability of the library plasmid to rescue the synthetic lethal phenotype.

3.5. Identification of the Synthetic Lethal Gene

Once a positive clone is identified, the insert sequence of the library plasmid can be determined by sequencing both ends of the insert and searching the *S. cerevisiae* Genome database at http://www.yeastgenome.org. If the insert contains more than one gene, a deletion analysis may be performed to determine which gene is capable of rescuing the synthetic lethal phenotype. Once this synthetic lethal gene is identified, a null or conditional mutation may be created in this gene and combined with the original gene of interest to see if cells carrying both mutations will be indeed inviable.

4. Notes

1. When choosing a strain with an *ade2* and *ade3* mutation, selecting a strain with a deletion of the gene rather than a point mutation will eliminate *ADE2* and *ADE3* revertants, thus helping to eliminate false-positives.
2. Better color development can be achieved by placing the plates at 4°C for a few days after colonies have grown. For better color development on SD medium, use half the concentration of adenine (final concentration of 10 µg/mL).
3. The mutagenesis protocol can be adapted to suit the needs of any mutagen. For more details, please *see* **Subheading 3.2.** in this chapter. Although some mutagens can be deactivated by addition of organic compounds, proper disposal of medium containing chemicals should be in accordance with local biosafety policies.
4. Some UV light sources will cast a shadow at the edge of the plate. Avoid spreading cells to the edges.
5. When plating the cells, try to dilute enough to allow for large colonies to form. Plating a high density of cells results in smaller colonies, making the selection of sectoring colonies difficult.
6. Each putative synthetic lethal mutant should be checked to ensure the color phenotype is a result of a second mutation and not reversion of marker genes. If the solid red colony is owing to reversion of marker gene, the cells will be able to grow on FOA plates, whereas the true synthetic mutant is unable to grow.

Acknowledgments

The authors wish to thank Michelle Hanna for proofreading the manuscript. This work is supported by the Canadian Institutes of Health Research operating grant MOP-38104 to WX.

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17

Synthetic Genetic Array Analysis in *Saccharomyces cerevisiae*

Amy Hin Yan Tong and Charles Boone

Summary

Synthetic lethality occurs when the combination of two mutations leads to an inviable organism. Screens for synthetic lethal genetic interactions have been used extensively to identify genes whose products buffer one another or impinge on the same essential pathway. For the yeast *Saccharomyces cerevisiae*, we developed a method termed Synthetic Genetic Array (SGA) analysis, which offers an efficient approach for the systematic construction of double mutants and enables a global analysis of synthetic lethal genetic interactions. In a typical SGA screen, a query mutation is crossed to an ordered array of approx 5000 viable gene deletion mutants (representing ~80% of all yeast genes) such that meiotic progeny harboring both mutations can be scored for fitness defects. This array-based approach automates yeast genetic analysis in general and can be easily adapted for a number of different screens, including genetic suppression, plasmid shuffling, dosage lethality, or suppression.

Key Words: Yeast; genetics; synthetic lethal; SGA; deletion mutant; double mutant; genetic interaction network; suppression; plasmid shuffling; dosage lethality; dosage suppression.

1. Introduction

Genetic analysis is important for assessing the biological roles of genes in vivo and remains a powerful tool for identifying new components of specific pathways and for ordering the function of gene products within a pathway. A combination of mutations in two genes that results in death or reduced fitness is termed a synthetic lethal or synthetic sick interaction, respectively ***(1)***. Synthetic lethality has been used extensively in different model organisms to identify genes whose products buffer one another and impinge on the same essential process ***(2–4)***.

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

For the budding yeast *Saccharomyces cerevisiae*, an international consortium of laboratories generated a collection of gene deletion mutants for each of the approx 6000 predicted genes, identifying approx 1000 essential genes and creating approx 5000 viable deletion mutants ***(5,6)***. The introduction of molecular tags or barcodes, a unique 20-bp DNA sequence at either end of the deletion cassette, identifies each gene deletion strain and enables the fitness of a particular mutant to be assessed within a population using a barcode microarray ***(7)***. The collection of approx 5000 viable deletion mutants provided the first opportunity for systematic genetic analysis in yeast and the potential for examining 12.5 million different double mutants for a synthetic lethal or sick phenotype.

Synthetic genetic array (SGA) analysis enables the systematic construction of double mutants ***(8,9)***, allowing large-scale mapping of synthetic genetic interactions. A typical SGA analysis involves crossing a query strain to the array of approx 5000 viable deletion mutants, and through a series of replica-pinning procedures, the double mutants are selected and scored for growth defects. Applying SGA analysis to 132 query mutations enabled us to generate a genetic interaction network containing approx 1000 genes and approx 4000 interactions, with functional information associated with the position and connectivity of a gene on the network.

The SGA methodology is quite versatile because any genetic element (or any number of genetic elements) marked by a selectable marker(s) can be manipulated similarly. In this regard, SGA methodology automates yeast genetics generally, such that specific alleles of genes, including point mutants and temperature-sensitive alleles, or plasmids can be crossed into any ordered array of strains providing systematic approaches to genetic suppression analysis, dosage lethality, dosage suppression, or plasmid shuffling. In this chapter, we describe the steps of SGA analysis in detail and hope to encourage other laboratories to adopt this methodology to suit their specific fields.

2. Materials

2.1. Media and Stock Solutions

1. G418 (Geneticin, Invitrogen): Dissolve in water at 200 mg/mL, filter-sterilize, and store in aliquots at 4°C.
2. clonNAT (nourseothricin, Werner BioAgents, Jena, Germany): Dissolve in water at 100 mg/mL, filter-sterilize, and store in aliquots at 4°C.
3. Canavanine (L-canavanine sulfate salt; Sigma): Dissolve in water at 100 mg/mL, filter-sterilize, and store in aliquots at 4°C.
4. Thialysine (*S*-[2-aminoethyl]-L-cysteine hydrochloride; Sigma): Dissolve in water at 100 mg/mL, filter-sterilize, and store in aliquots at 4°C.
5. Amino-acids supplement powder mixture for synthetic media (complete): Contains 3 g adenine (Sigma), 2 g uracil (ICN), 2 g inositol, 0.2 g para-aminobenzoic

acid (Acros Organics), 2 g alanine, 2 g arginine, 2 g asparagine, 2 g aspartic acid, 2 g cysteine, 2 g glutamic acid, 2 g glutamine, 2 g glycine, 2 g histidine, 2 g isoleucine, 10 g leucine, 2 g lysine, 2 g methionine, 2 g phenylalanine, 2 g proline, 2 g serine, 2 g threonine, 2 g tryptophan, 2 g tyrosine, 2 g valine (Fisher). Drop-out (DO) powder mixture is a combination of the aforementioned ingredients minus the appropriate supplement. 2 g of the DO powder mixture is used per liter of medium (*see* **Note 1**).

6. Amino-acids supplement for sporulation medium: Contains 2 g histidine, 10 g leucine, 2 g lysine, 2 g uracil; 0.1 g of the amino-acid supplements powder mixture is used per liter of sporulation medium (*see* **Note 1**).
7. β-glucuronidase (Sigma): Prepare 0.5% solution in water and store at 4ºC.
8. Glucose (Dextrose, Fisher): Prepare 40% solution, autoclave, and store at room temperature.
9. YEPD: Add 120 mg adenine (Sigma), 10 g yeast extract, 20 g peptone, 20 g bacto agar (BD Difco) to 950 mL water in a 2-L flask. After autoclaving, add 50 mL of 40% glucose solution, mix thoroughly, cool to approx 65°C, and pour plates.
10. YEPD + G418: Cool YEPD medium to approx 65°C, add 1 mL of G418 stock solution (final concentration 200 mg/L), mix thoroughly, and pour plates.
11. YEPD + clonNAT: Cool YEPD medium to approx 65°C, add 1 mL of clonNAT stock solution (final concentration 100 mg/L), mix thoroughly, and pour plates.
12. YEPD + G418/clonNAT: Cool YEPD medium to approx 65°C, add 1 mL of G418 (final concentration 200 mg/L), and 1 mL of clonNAT (final concentration 100 mg/L) stock solutions, mix thoroughly, and pour plates.
13. Enriched sporulation: Add 10 g potassium acetate (Fisher), 1 g yeast extract, 0.5 g glucose, 0.1 g amino-acids supplement powder mixture for sporulation, 20 g bacto agar to 1 L water in a 2-L flask. After autoclaving, cool medium to approx 65°C, add 250 µL of G418 stock solution (final concentration 50 mg/L), mix thoroughly, and pour plates.
14. (SD/MSG) – His/Arg/Lys + canavanine/thialysine/G418: Add 1.7 g yeast nitrogen base without amino acids or ammonium sulfate (BD Difco), 1 g MSG (L-glutamic acid sodium salt hydrate; Sigma), 2 g amino-acids supplement powder mixture (DO – His/Arg/Lys), 100 mL water in a 250-mL flask. Add 20 g bacto agar to 850 mL water in a 2-L flask. Autoclave separately. Combine autoclaved solutions, add 50 mL 40% glucose, cool medium to approx 65°C, add 0.5 mL canavanine (50 mg/L), 0.5 mL thialysine (50 mg/L), and 1 mL G418 (200 mg/L) stock solutions, mix thoroughly, and pour plates (*see* **Note 2**).
15. (SD/MSG) – His/Arg/Lys + canavanine/thialysine/clonNAT: Add 1.7 g yeast nitrogen base without amino acids or ammonium sulfate, 1 g MSG, 2 g amino-acids supplement powder mixture (DO – His/Arg/Lys), 100 mL water in a 250-mL flask. Add 20 g bacto agar to 850 mL water in a 2-L flask. Autoclave separately. Combine autoclaved solutions, add 50 mL 40% glucose, cool medium to approx 65°C, add 0.5 mL canavanine (50 mg/L), 0.5 mL thialysine (50 mg/L), and 1 mL clonNAT (100 mg/L) stock solutions, mix thoroughly, and pour plates.
16. (SD/MSG) – His/Arg/Lys + canavanine/thialysine/G418/clonNAT: Add 1.7 g yeast nitrogen base without amino acids or ammonium sulfate, 1 g MSG, 2 g

amino-acids supplement powder mixture (DO – His/Arg/Lys), 100 mL water in a 250-mL flask. Add 20 g bacto agar to 850 mL water in a 2-L flask. Autoclave separately. Combine autoclaved solutions, add 50 mL 40% glucose, cool medium to approx 65°C, add 0.5 mL canavanine (50 mg/L), 0.5 mL thialysine (50 mg/L), 1 mL G418 (200 mg/L), and 1 mL clonNAT (100 mg/L) stock solutions, mix thoroughly, and pour plates.

17. (SD/MSG) Complete: Add 1.7 g yeast nitrogen base without amino acids or ammonium sulfate, 1 g MSG, 2 g amino-acids supplement powder mixture (complete), 100 mL water in a 250-mL flask. Add 20 g bacto agar to 850 mL water in a 2-L flask. Autoclave separately. Combine autoclaved solutions, add 50 mL of 40% glucose, mix thoroughly, cool medium to approx 65°C, and pour plates.
18. SD – His/Arg/Lys + canavanine/thialysine: Add 6.7 g yeast nitrogen base without amino acids (BD Difco), 2 g amino-acids supplement powder mixture (DO – His/Arg/Lys), 100 mL water in a 250-mL flask. Add 20 g bacto agar to 850 mL water in a 2-L flask. Autoclave separately. Combine autoclaved solutions, add 50 mL 40% glucose, cool medium to approx 65°C, add 0.5 mL canavanine (50 mg/L), and 0.5 mL thialysine (50 mg/L) stock solutions, mix thoroughly, and pour plates (*see* **Note 3**).
19. SD – Leu/Arg/Lys + canavanine/thialysine: Add 6.7 g yeast nitrogen base w/o amino acids, 2 g amino-acids supplement powder mixture (DO – Leu/Arg/Lys), 100 mL water in a 250-mL flask. Add 20 g bacto agar to 850 mL water in a 2-L flask. Autoclave separately. Combine autoclaved solutions, add 50 mL 40% glucose, cool medium to approx 65°C, add 0.5 mL canavanine (50 mg/L), and 0.5 mL thialysine (50 mg/L) stock solutions, mix thoroughly, and pour plates.

2.2. Plates and Accessories

1. OmniTrays (Nunc, cat. no. 242811) are used for SGA analysis (*see* **Note 4**).
2. 60-mm dishes (Fisher) are used for random spore analysis (*see* **Note 5**).
3. Aluminum sealing tape (Nunc, cat. no. 276014) is used for resealing the 96-well plates that contain the frozen stocks of yeast deletion strains.

2.3. Manual Pin Tools

The following manual pin tools can be purchased from V & P Scientific, Inc. (San Diego, CA).

1. 96 floating pin E-clip style manual replicator (VP408FH).
2. 384 floating pin E-clip style manual replicator (VP384F).
3. For extra floating pins (FP): 1.58 mm diameter with chamfered tip (*see* **Note 6**).
4. Registration accessories: Library Copier™ (VP381), Colony Copier™ (VP380).
5. Pin-cleaning accessories: plastic bleach or water reservoirs (VP421), pyrex alcohol reservoir with lid (VP420), pin-cleaning brush (VP425) (*see* **Note 7**).

2.4. Robotic Pinning Systems

1. VersArray colony arrayer system (BioRad Laboratories).
2. QBot, QPixXT, MegaPix (Genetix, Boston, MA).
3. Singer Rotor HDA bench top robot (Singer Instruments, Somerset, UK) (*see* **Note 4**).

2.5. Strains and Plasmids

1. Six different starting strains were constructed and used in the SGA screens (*see* **Table 1**). Y5563 (*MATα can1Δ::MFA1pr-HIS3 lyp1Δ ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*) and Y5565 (*MATα can1Δ::MFA1pr-HIS3 mfα1Δ::MFα1pr-LEU2 lyp1Δ ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*) are the current starting strains for the construction of an SGA query strain (*see* **Note 8**).
2. p4339 (pCRII-TOPO::natRMX4; *see* **Note 9**).
3. Y7221 (*MATα can1Δ::MFA1pr-HIS3 lyp1Δ cyh2 ura3Δ::natR leu2Δ0 his3Δ1 met15Δ0*) is the wild-type control strain for the *natR*-marked query strains.
4. The collection of *MAT***a** deletion strains can be purchased from Invitrogen (http://www.invitrogen.com) as stamped 96-well agar plates, American Type Culture Collection (http://www.atcc.org/cydac/cydac.cfm) as stamped 96-well agar plates, EUROSCARF (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html) as stamped 96-well agar plates, and Open Biosystems (http://www.openbiosystems.com/yeast_collections.php) as stamped 96-well agar plates or frozen stocks in 96-well plates.

3. Methods

3.1. SGA Query Strain Construction

3.1.1. Nonessential Genes: PCR-Mediated Gene Deletion

1. Two gene-deletion primers are synthesized, each containing 55 bp of sequence at the 5' end that is specific to the region upstream or downstream of the gene of interest (*Gene X*), excluding the start and stop codons, and 22 bp of sequence at the 3' end that is specific for the amplification of the *natMX4 (10)* cassette (**Fig. 1A** and **Table 2**).
2. The *natMX4* cassette flanked with 55 bp target sequences is amplified from p4339 with the gene-deletion primers designed in **step 1** (*see* **Note 10**).
3. Transform the polymerase chain reaction (PCR) product into the SGA starting strain, Y5563. Select transformants on YEPD + clonNAT medium.
4. Verify correct targeting of the deletion cassette by PCR.

3.1.2. Nonessential Genes: Switching Method

1. Obtain the deletion strain of interest (*xxxΔ::kanR*) from the *MAT***a** deletion collection and mate with Y5565, isolate diploid zygotes by micromanipulation (**Fig. 2**).

Table 1
Yeast Strains

Strain	Genotype	Source
Y2454	*MATα mfa1Δ::MFA1pr-HIS3 can1Δ ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*	**ref. *8***
Y3068	*MATα can1Δ::MFA1pr-HIS3 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 lys2Δ0*	**ref. *8***
Y3084	*MATα can1Δ::MFA1pr-HIS3 mfα1Δ::MFα1pr-LEU2 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 lys2Δ0*	**ref. *9***
Y3656	*MATα can1Δ::MFA1pr-HIS3-MFα1pr-LEU2 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 lys2Δ0*	**ref. *9***
Y5563	*MATα can1Δ::MFA1pr-HIS3 lyp1Δ ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*	Boone Lab
Y5565	*MATα can1Δ::MFA1pr-HIS3 mfa1Δ::MFα1pr-LEU2 lyp1Δ ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*	Boone Lab
Y7221	*MATα can1Δ::MFA1pr-HIS3 lyp1Δ ura3Δ0::natR leu2Δ0 his3Δ1 met15Δ0 cyh2*	Boone Lab

2. Transform the resulting diploid with *Eco*RI-cut p4339, which switches the gene deletion marker from *kanMX* to *natMX*. Select transformants on YEPD + clonNAT medium.
3. Transfer the resultant diploids to enriched sporulation medium, incubate at 22°C for 5 d.
4. Resuspend a small amount of spores in sterile water, and plate on SD – Leu/Arg/Lys + canavanine/thialysine to select *MATα* meiotic progeny; incubate at 30°C for approx 2 d (*see* **Note 11**).
5. Replica plate to YEPD + clonNAT to identify the *MATα* meiotic progeny that carry the query deletion marked with *natMX* (*xxxΔ::natR*).

3.1.3. Essential Genes: PCR-Mediated Integration of Conditional Allele

1. Two pairs of oligonucleotides are synthesized. The first pair of primers is used in the amplification of the conditional allele of interest (*gene x**), including 200 bp downstream of its stop codon, such that the reverse primer contains an additional 25 bp complementary sequence to the *natMX4* cassette at the 5' end (**Fig. 1B**). The second pair of primers is used in the amplification of the *natMX4* cassette, such that the reverse primer contains a 45 bp complementary sequence downstream of the target gene (*Gene X*).

Fig. 1. *(opposite page)* Strategies of construction of the SGA query strain. **(A)** PCR-mediated gene deletion is used to construct a nonessential query strain. The lines outside of the boxes represent the primers used for the PCR reaction. The thicker lines represent the primer sequences that anneal to the *natMX4* cassette (*see* **Table 2**). The thinner lines represent the 55 bp sequence specific to the upstream or downstream sequences of the target gene (*Gene X*). The *natMX4* cassette with flanking sequences is amplified and

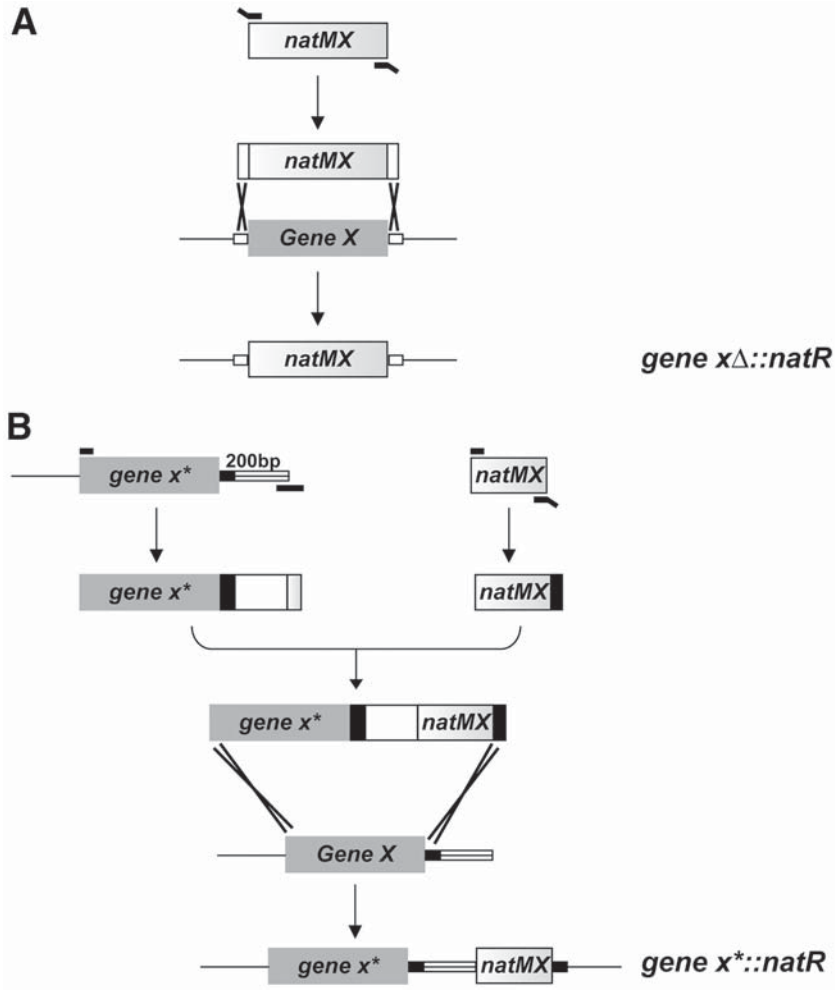


Fig. 1. *(continued)* transformed into the SGA starting strain, Y5563. Gene deletion is mediated by homologous recombination between the ends of the target sequences of the PCR product and the corresponding genomic DNA sequence. Transformants carrying the target gene deletion (*gene xΔ::natR*) are selected on YEPD + clonNAT. **(B)** Two-step PCR-mediated gene integration is used to construct a conditional allele query strain. First, the conditional allele and the marker are amplified separately. The conditional allele of interest (*gene x**) and 200 bp downstream of its stop codon is amplified using primers to create a fragment that overlaps with the *natMX4* cassette. The *natMX4* cassette is amplified using primers to create a fragment that overlaps with the immediate downstream sequence of the target gene. Second, the PCR products are combined and co-transformed into the SGA starting strain, Y5563. Transformants are selected on YEPD + clonNAT under the permissive condition. Correct integration of the conditional allele (*gene x*::natR*) is identified by replica plating to the restrictive condition.

Table 2
Primer Sequences

Primer	Sequence 5' to 3'	Comments
MX-F	ACATGGAGGCCCAGAATACCCT	MX-cassette amplification
MX-R	CAGTATAGCGACCAGCATTCAC	MX-cassette amplification

2. Mix the two PCR products together and transform into the SGA starting strain, Y5563. Select transformants on YEPD + clonNAT medium.
3. Verify correct targeting of the conditional allele by replica plating to the restrictive condition.

3.2. Sterilization Procedure for the Pin Tools

3.2.1. Manual Pin Tools

1. Set up the wash reservoirs as follows: three trays of sterile water of increasing volume: 30 mL, 50 mL, and 70 mL, one tray of 40 mL of 10% bleach, one tray of 90 mL of 95% ethanol (*see* **Note 12**).
2. Let the replicator sit in the 30-mL water reservoir for approx 1 min to remove the cells on the pins.
3. Place the replicator in 10% bleach for approx 20 s.
4. Transfer the replicator to the 50-mL water reservoir and then to the 70-mL water reservoir to rinse the bleach off the pins.
5. Transfer the replicator to 95% ethanol.
6. Let excess ethanol drip off the pins, then flame.
7. Allow replicator to cool (*see* **Note 13**).

3.2.2. Robotic Pin Tools (VersArray colony arrayer system)

Use the following procedure to clean and sterilize the pins prior to starting on the robot.

1. Fill the sonicator with 230 mL of sterile water.
2. Clean the replicator in the sonicator for 5 min.
3. Remove the water, fill the sonicator with 230 mL of 75% ethanol.
4. Sterilize the replicator in the sonicator for 5 min.
5. Let the replicator sit in a tray of 90 mL of 95% ethanol for 30 s.
6. Allow the replicator to dry over the fan for 30 s.

Fig. 2. *(opposite page)* Construction of the SGA query strain using the switching method. The *MAT***a** deletion strain of interest (*xxxΔ::kanR*) is crossed to the *MAT*α switcher strain, Y5565. The resultant diploid is transformed with *Eco*RI-cut p4339 to switch the gene deletion marker from *kanMX* to *natMX*. The resultant diploid is transferred to medium with

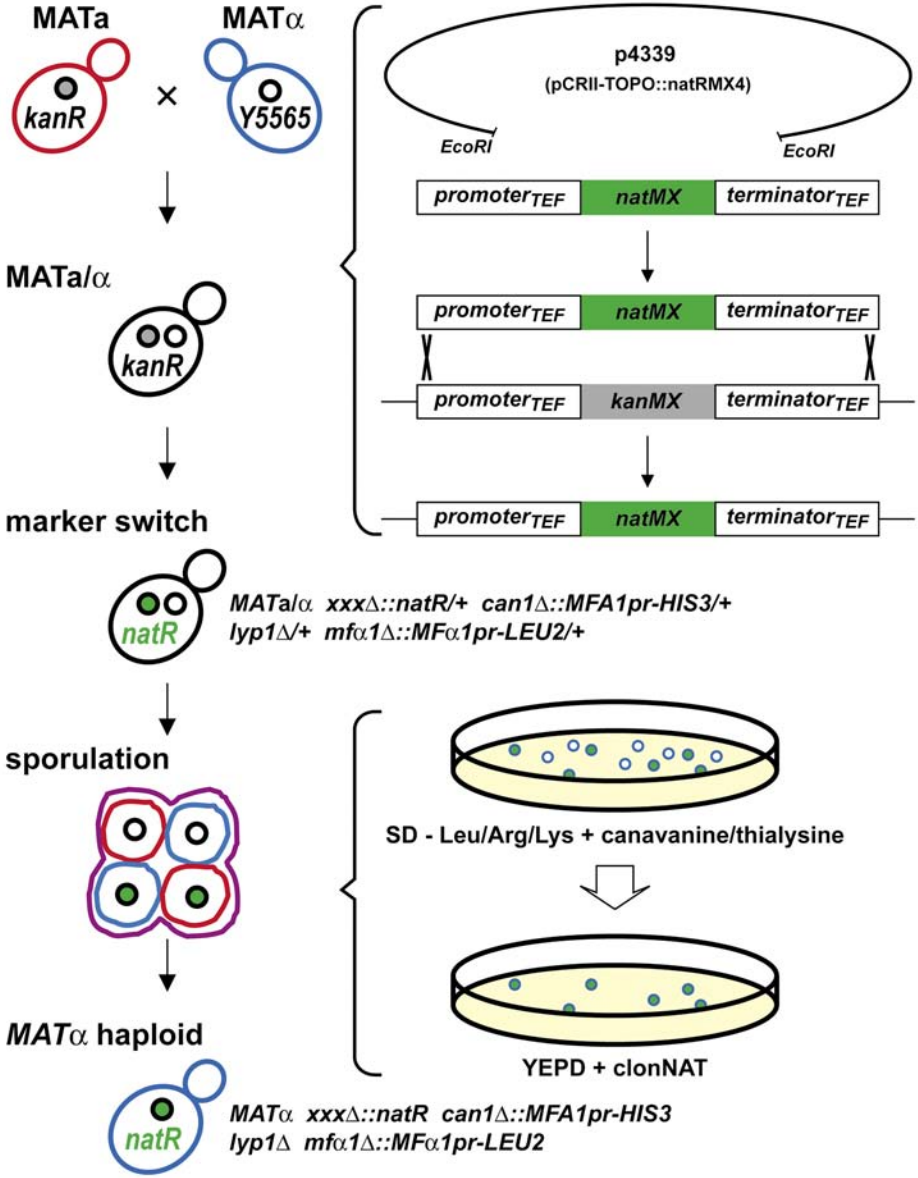


Fig. 2. *(continued)* reduced levels of carbon and nitrogen to induce sporulation and the formation of haploid meiotic spore progeny. Spores are transferred to synthetic medium lacking leucine, which allows for selective germination of *MAT*α meiotic progeny because only these cells express the *MFα1pr-LEU2* reporter; and containing canavanine and thialysine, which allows for selective germination of meiotic progeny that carries the *can1Δ* (*can1Δ::MFA1pr-HIS3*) and *lyp1Δ* markers. The *MAT*α meiotic progeny are then replica plated to medium that contains clonNAT, which selects for growth of meiotic progeny that carries the gene deletion mutation (*xxxΔ::natR*).

Use the following procedure to sterilize the pins at the end of each replica-pinning step.

1. Set up the wash reservoirs as follows: two trays of sterile water of 50 mL and 60 mL, respectively, a tray of 90 mL of 95% ethanol, and the sonicator with 230 mL of 75% ethanol.
2. Let the replicator sit in the 50-mL water reservoir for 1 min to remove the cells on the pins.
3. Let the replicator sit in the 60-mL water reservoir for 1 min to remove the cells on the pins.
4. Sterilize the replicator in the 70% ethanol-sonicator for 2 min.
5. Let the replicator sit in the 95% ethanol for 30 s.
6. Allow the replicator to dry over the fan, for 30 s.

3.3. Building a 768-Density Deletion Mutant Array Using the Manual Pin Tools

1. Peel off the foil coverings slowly on the frozen 96-well microtiter plates.
2. Let the plates thaw completely on a flat surface.
3. Mix the glycerol stocks gently by stirring with a 96-pin replicator.
4. Replicate the glycerol stocks from the 96-well plates onto YEPD + G418 agar plates using the Library Copier™ with the pair of one-alignment holes on the front frame (**Fig. 3A**). Take extreme caution that the pins do not drip liquid into neighboring wells.
5. Reseal the 96-well plates with fresh aluminum sealing tape and return to –80°C.
6. Let cells grow at room temperature for approx 2 d.
7. Condense four plates of 96-format into one plate of 384-format using the 96-pin replicator and the Library Copier with the pair of four-alignment holes on the front frame (**Fig. 3B**).
8. Let cells grow at room temperature for approx 2 d (*see* **Note 14**).
9. Replicate the 384 strains onto a fresh plate with a 384-pin replicator and the Library Copier with the pair of four-alignment holes on the front frame. Use alignment holes "A" and "D" to create the working density of 768 (i.e., duplicates of 384 mutants).
10. Let cells grow at room temperature for approx 2 d, to generate the working copy of the deletion mutant array (DMA).

3.4. SGA Analysis

3.4.1. SGA Procedure

Figure 4 shows the selection steps in the SGA analysis.
Query Strain and DMA.

1. Grow the query strain in a 5-mL overnight culture in YEPD.
2. Pour the query strain culture over a YEPD plate, use the replicator to transfer the liquid culture onto two fresh YEPD plates, generating a source of newly grown

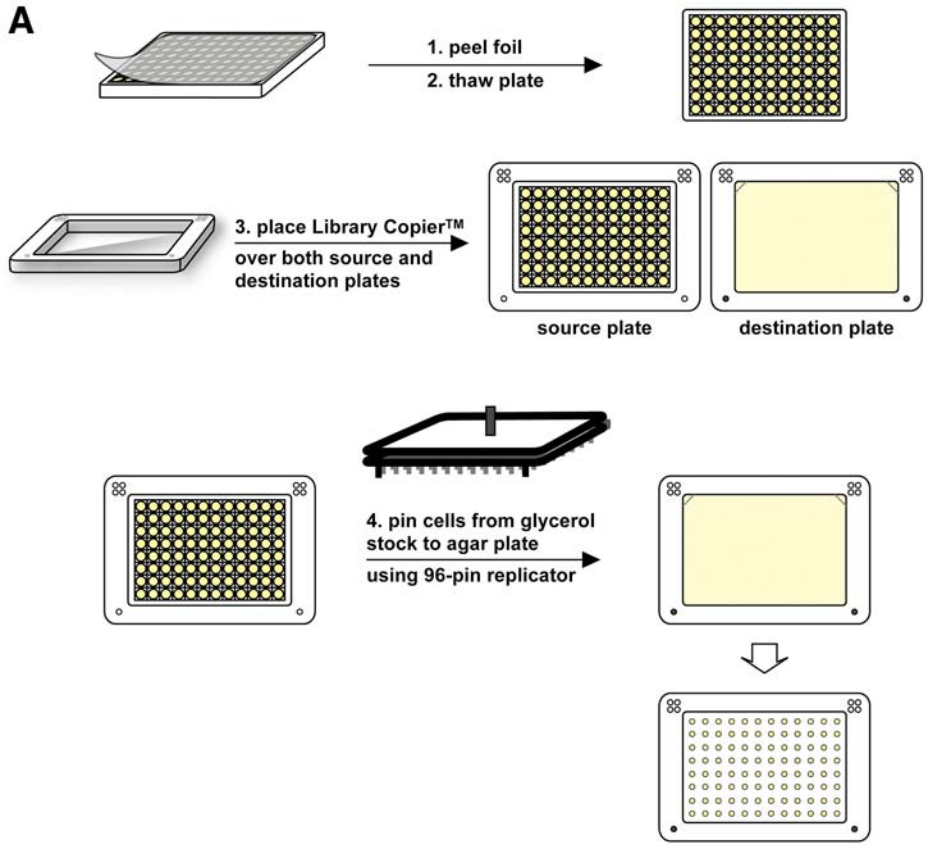


Fig. 3. Building a 768-density deletion mutant array (DMA) using the manual pin tools. Glycerol stocks are pinned to an agar plate using a 96-pin replicator and Library Copier with the pair of one-alignment holes on the front frame as depicted **(A)**.

query cells for mating to the DMA in the density of 768. Let cells grow at 30°C for 1 d (*see* **Note 15**).

3. Replicate the DMA to fresh YEPD + G418. Let cells grow at 30°C for 1 d (*see* **Note 16**).

Mating the Query Strain with the DMA.

4. Pin the 768-format query strain onto a fresh YEPD plate.
5. Pin the DMA on top of the query cells.
6. Incubate the mating plates at room temperature for 1 d.

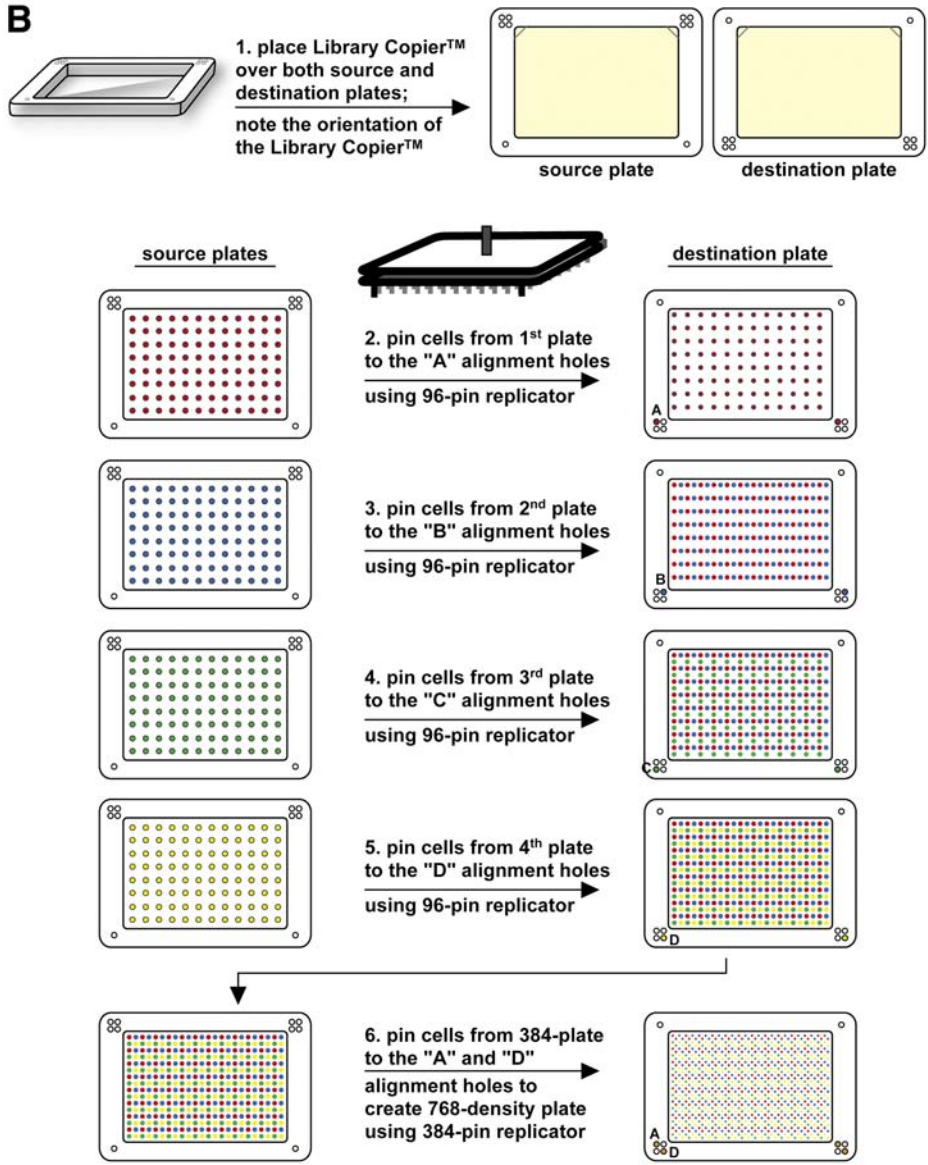


Fig. 3. Building a 768-density deletion mutant array (DMA) using the manual pin tools. Four 96-plates are condensed to form one 384-plate using the 96-pin replicator and Library Copier with the pair of four-alignment holes on the front frame as depicted **(B)**. Finally, the 384 strains are transferred to a fresh plate using a 384-pin replicator and by registering the guide pins into alignment hole "A" of the Library Copier; this step is repeated but the guide pins are registered into alignment hole "D" of the Library Copier.

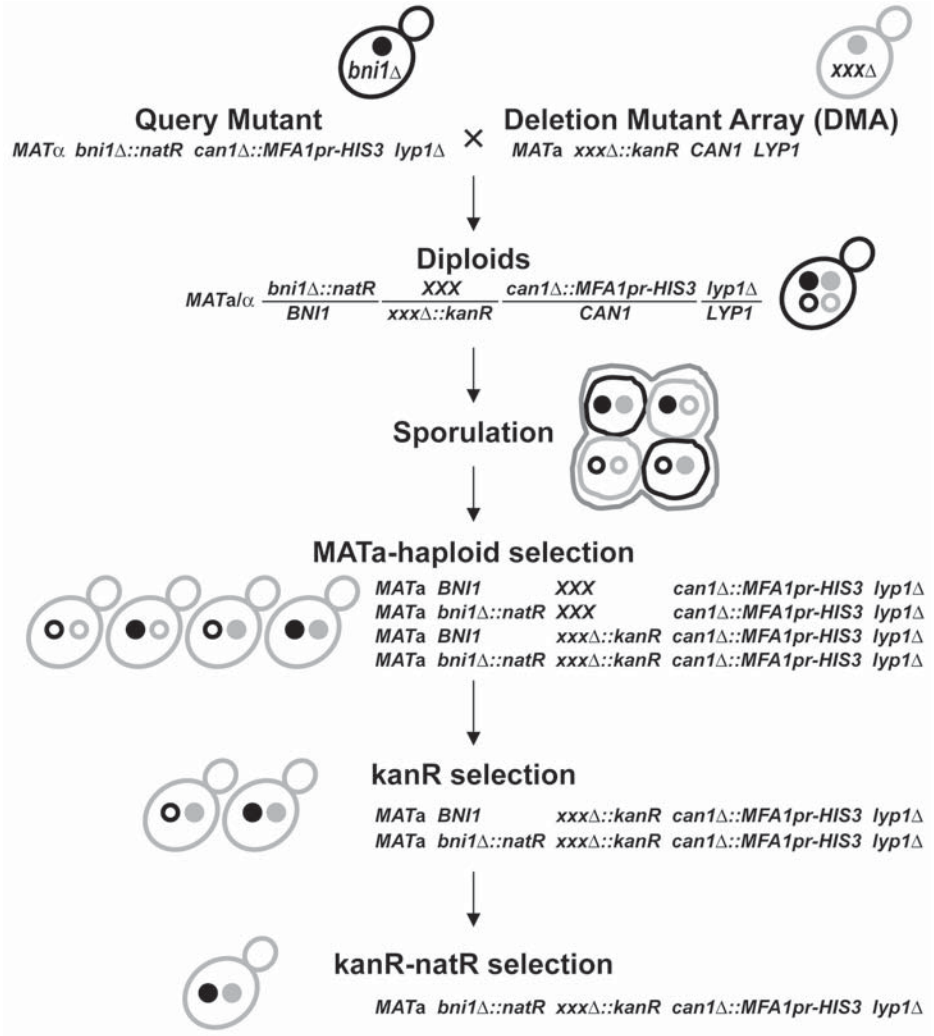


Fig. 4. Synthetic genetic array (SGA) methodology. A *MAT*α strain carrying a query mutation (*bni1Δ*) linked to a dominant selectable marker, such as the nourseothricin-resistance marker *natMX* that confers resistance to the antibiotic nourseothricin (clonNAT), and the *MFA1pr-HIS3*, *can1Δ*, and *lyp1Δ* reporters is crossed to an ordered array of *MAT***a** viable yeast deletion mutants, each carrying a gene deletion mutation linked to a kanamycin-resistance marker *kanMX* that confers resistance to the antibiotic geneticin (G418). Growth of resultant zygotes is selected for on medium containing nourseothricin and geneticin. The heterozygous diploids are transferred to medium with reduced levels of carbon and nitrogen to induce sporulation and the formation of haploid meiotic spore progeny. Spores are transferred to synthetic medium lacking histidine, which allows for selective germination of *MAT***a** meiotic progeny

*MAT***a**/α Diploid Selection and Sporulation.

7. Pin the resulting *MAT***a**/α zygotes onto YEPD + G418/clonNAT plates.
8. Incubate the diploid-selection plates at 30°C for 2 d.
9. Pin diploid cells to enriched sporulation medium.
10. Incubate the sporulation plates at 22°C for 5 d (*see* **Note 17**).

*MAT***a** Meiotic Progeny Selection.

11. Pin spores onto SD – His/Arg/Lys + canavanine/thialysine plates.
12. Incubate the haploid-selection plates at 30°C for 2 d.
13. Pin the *MAT***a** meiotic progeny onto SD – His/Arg/Lys + canavanine, thialysine plates for a second round of haploid selection.
14. Incubate the plates at 30°C for 1 d.

*MAT***a**-kanR Meiotic Progeny Selection.

15. Pin the *MAT***a** meiotic progeny onto (SD/MSG) – His/Arg/Lys + canavanine/thialysine/G418 plates.
16. Incubate the kanR-selection plates at 30°C for 2 d.

*MAT***a**-kanR-natR Meiotic Progeny Selection.

17. Pin the *MAT***a** meiotic progeny onto (SD/MSG) – His/Arg/Lys + canavanine/thialysine/G418/clonNAT plates.
18. Incubate the kanR/natR-selection plates at 30°C for 2 d.
19. Score double mutants for fitness defect (*see* **Note 18**).

3.4.2. Scoring of Putative Interactions in an SGA Screen

1. Perform an SGA screen using the "wild-type" control strain (Y7221) following the steps as described in **Subheading 3.4.1.**
2. Visually inspect the experimental plates by comparing to the wild-type control plates, taking note of the double mutant colonies that fail to grow, or appear smaller in size (*see* **Note 19**).
3. Record the potential hits in the first-round screening.
4. Repeat the screen two more times, for a total of three independent screens.
5. Record the potential hits for the second- and third-round screenings.

Fig. 4. *(continued)* because only these cells express the *MFA1pr-HIS3* reporter; and containing canavanine and thialysine, which allows for selective germination of meiotic progeny that carries the *can1Δ* and *lyp1Δ* markers. The *MAT***a** meiotic progeny are then transferred to medium that contains G418, which selects for growth of meiotic progeny that carries the gene deletion mutation (*xxxΔ::kanR*). Finally, the *MAT***a** meiotic progeny are transferred to medium that contains both clonNAT and G418, which then selects for growth of double mutant (*bni1Δ::natR xxxΔ::kanR*).

6. Generate an unbiased set of putative interactions by including all those that appear two or three times in the three rounds of screening.
7. Generate a biased set of putative interactions by sorting the one-time hits according to the functional annotations such as Gene Ontology (GO) molecular function and biological process, and selecting those that are related functionally to multiple genes within the unbiased set (*see* **Note 20**).
8. Combine both sets of putative interactions to create a list for confirmation.

3.5. Confirmation of the Putative Interactions Generated From SGA Analysis

3.5.1. Random Spore Analysis

1. Inoculate a small amount of spores (approximately the size of a pinprick) in 1 mL of sterile water; mix well (*see* **Note 21**) ***(11)***.
2. Plate 20 µL on SD – His/Arg/Lys + canavanine/thialysine (*see* **Note 22**).
3. Plate 40 µL on (SD/MSG) – His/Arg/Lys + canavanine/thialysine/G418.
4. Plate 40 µL on (SD/MSG) – His/Arg/Lys + canavanine/thialysine/clonNAT.
5. Plate 80 µL on (SD/MSG) – His/Arg/Lys + canavanine/thialysine/G418/clonNAT.
6. Incubate the plates at 30°C for approx 1.5–2 d.
7. Score the double-drugs selection against the single-drug selections (**Fig. 5**).

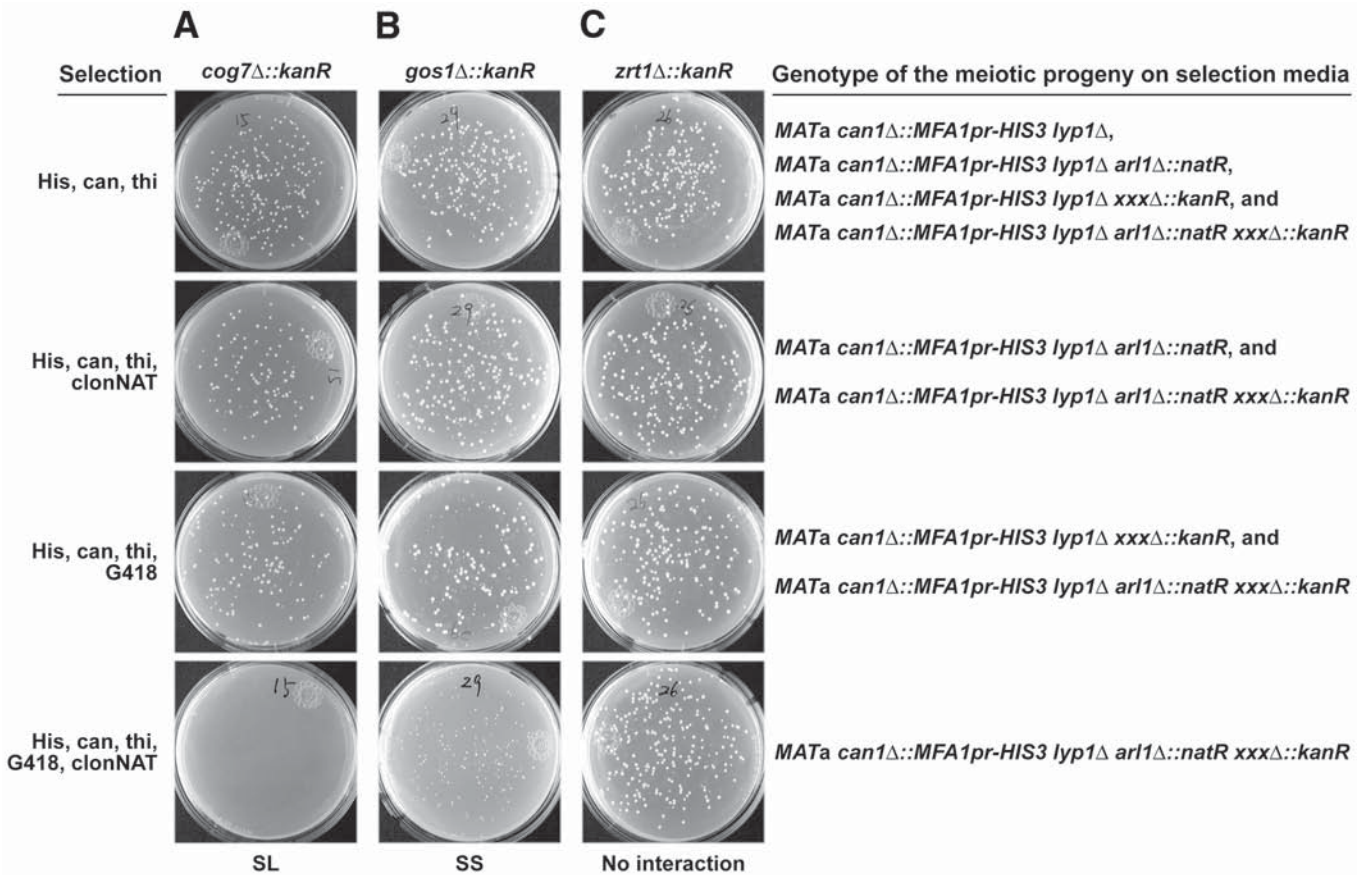
3.5.2. Tetrad Analysis

1. Inoculate a small amount of spores (approximately the size of a pinprick) in 100 µL of 0.5% β-glucuronidase solution.
2. Mix gently by stirring the loop and incubate at room temperature for 15 min.
3. Spread approx 30 µL of digested spores on (SD/MSG) Complete medium (*see* **Note 23**).
4. Dissect tetrads.

3.6. Applications of the SGA Methodology

To examine synthetic genetic interactions with the essential genes, an SGA query strain can be crossed to the Tet-promoters Hughes collection (yTHC) (Open Biosystems), double mutants can be selected and scored for growth defects in the presence of doxycycline, which downregulates the expression of the essential genes ***(12)***.

The SGA methodology can be easily extended to other forms of genetic interactions, for example, higher-order genetic interactions (triple mutant genetic interactions) ***(9)***, dosage lethality, and suppression using high-copy plasmid or regulatory expression of yeast genes or heterologous genes. Reporter constructs, such as *SCB::HIS3* ***(13)***, can be incorporated into the SGA methodology to monitor specific transcriptional responses in the approx 5000 deletion mutant backgrounds. A Yeast Overexpression Array, containing


A
B
C
Selection
cog7Δ::kanR
gos1Δ::kanR
zrt1Δ::kanR
Genotype of the meiotic progeny on selection media
His, can, thi
MATa can1Δ::MFA1pr-HIS3 lyp1Δ,
MATa can1Δ::MFA1pr-HIS3 lyp1Δ arl1Δ::natR,
MATa can1Δ::MFA1pr-HIS3 lyp1Δ xxxΔ::kanR, and
MATa can1Δ::MFA1pr-HIS3 lyp1Δ arl1Δ::natR xxxΔ::kanR
His, can, thi, clonNAT
MATa can1Δ::MFA1pr-HIS3 lyp1Δ arl1Δ::natR, and
MATa can1Δ::MFA1pr-HIS3 lyp1Δ arl1Δ::natR xxxΔ::kanR
His, can, thi, G418
MATa can1Δ::MFA1pr-HIS3 lyp1Δ xxxΔ::kanR, and
MATa can1Δ::MFA1pr-HIS3 lyp1Δ arl1Δ::natR xxxΔ::kanR
His, can, thi, G418, clonNAT
MATa can1Δ::MFA1pr-HIS3 lyp1Δ arl1Δ::natR xxxΔ::kanR
SL
SS
No interaction

approx 6000 ORFs, has been assembled and can be used to screen for synthetic dosage lethality and suppression (R. Sopko, M. Snyder, C. Boone, and B. Andrews, unpublished data). Because double mutants are created by meiotic recombination, a set of gene deletions that is linked to the query gene, which we refer to as the "linkage group" form double mutants at a reduced frequency, thus, appearing synthetic lethal/sick with the query mutation. Because the gene deletions represent mapping markers covering all chromosomes in the yeast genome, SGA mapping (SGAM) has been shown as a method for high-resolution genetic mapping ***(14)***.

4. Notes

1. When making up the amino acids supplement mixture, the solid ingredients should be combined and then mixed thoroughly by turning end-over-end for at least 15 min. The resultant mixture can be stored in tinted glass bottles at room temperature.
2. Because ammonium sulfate impedes the function of G418 and clonNAT, synthetic medium containing these antibiotics is made with monosodium glutamic acid as a nitrogen source ***(15)***.
3. Because this medium does not contain any antibiotics such as G418 and clonNAT, ammonium sulfate is used as the nitrogen source.
4. The Singer Rotor DHA bench top robot uses disposable replicators (RePads), and larger surface area plates that have the same external footprint dimensions as OmniTray, PlusPlates.
5. We use OmniTrays for all the replica pinning steps involved in SGA analysis, 100-mm petri dishes for the construction of SGA query strains and tetrad analysis, and 60-mm Petri dishes for random spore analysis. We found that approx 35 mL of media in an OmniTray gives the optimal result. Excess media might cause uneven transfer of cells during replica-pinning, such as the pins poking through the agar along the edges. For random spore analysis, approx 10 mL of media in a 60-mm dish is optimal.

Fig. 5. *(opposite page)* Examples of the random spore analysis: *MAT***a** meiotic progeny derived from sporulation of heterozygous diploids, *MAT***a**/α *arl1Δ::natR*/+ *cog7Δ::kanR*/+ (A), *MAT***a**/α *arl1Δ::natR*/+ *gos1Δ::kanR*/+ (B), and *MAT***a**/α *arl1Δ::natR*/+ *zrt1Δ::kanR*/+ (C), plated onto media (SD – His/Arg/Lys + canavanine/thialysine), ([SD/MSG] – His/Arg/Lys + canavanine/thialysine/clonNAT), ([SD/MSG] – His/Arg/Lys + canavanine/thialysine/G418), ([SD/MSG] – His/Arg/Lys + canavanine/thialysine/G418/clonNAT) as indicated. The plates were incubated at 30°C for approx 2 d. Cell growth under the four conditions was compared and scored. The *MAT***a** *arl1Δ::natR cog7Δ::kanR* double mutant (A) was scored as having a synthetic lethal (SL) interaction. The *MAT***a** *arl1Δ::natR gos1Δ::kanR* double mutant (B) was scored as having a synthetic sick (SS) interaction. The *MAT***a** *arl1Δ::natR zrt1Δ::kanR* double mutant (C) was scored as having no interaction (No).

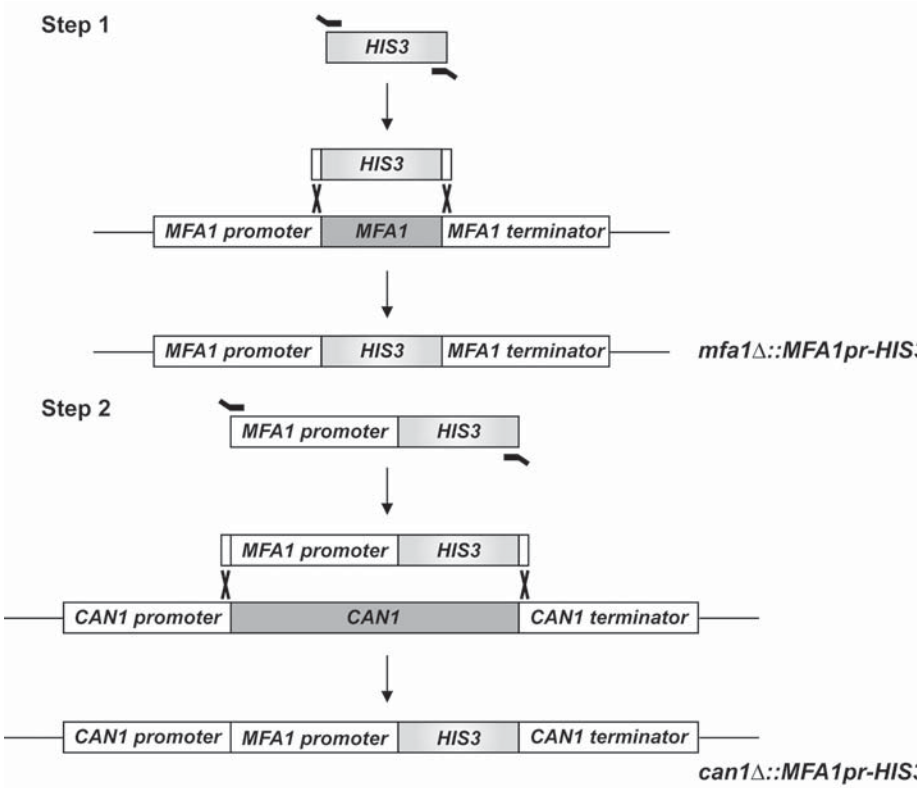


Fig. 6. Construction of the starting strain. The construction of *can1Δ::MFA1pr-HIS3* involves two steps. First, the *HIS3* opening reading frame (ORF) is integrated at the *MFA1* locus such that its expression is regulated by the *MFA1* promoter (*MFA1pr*), *mfa1Δ::MFA1pr-HIS3*. Second, *mfa1Δ::MFA1pr-HIS3* is integrated at the *CAN1* locus, replacing the *CAN1* gene, *can1Δ::MFA1pr-HIS3*.

6. The 1.58-mm diameter, flat-tip pins (FP6) can be used as an alternative to the chamfered-tip pins. They transfer more cells than the chamfered-tip pins, and might not be suitable for producing high-density arrays (768 spots/array).
7. Empty tip boxes can be used as a substitute to the reservoirs for bleach, water, and ethanol.
8. In Y5565, *LEU2* was integrated at the *MFα1* locus such that its expression is regulated by the *MFα1* promoter (*MFα1pr*), *mfα1Δ::MFα1pr-LEU2*. In both Y5563 and Y5565, *MFA1pr-HIS3* was integrated at the *CAN1* locus, *can1Δ::MFA1pr-HIS3* (**Fig. 6**). In addition, they differ from the previous starting strains, because they carry a *lyp1* marker that confers resistance to thialysine. To create an SGA query strain by PCR-mediated integration or gene disruption, we use Y5563 (*MATα can1Δ::MFA1pr-HIS3 lyp1Δ ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*). To create an SGA query strain by the switching method, we use Y5565

(*MATα can1Δ::MFA1pr-HIS3 mfα1Δ::MFα1pr-LEU2 lyp1Δ ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*).

9. Plasmid p4339 serves as a DNA template to amplify the *natRMX4* cassette required for PCR-mediated integration or gene deletion. It also serves as a *kanMX* to *natMX* maker-switcher plasmid.
10. Adding 5% dimethyl sulfoxide (DMSO) to the PCR reaction increases the product yield of the *natMX4* cassette.
11. To facilitate the selection of *MATα* meiotic progeny that carries the query mutation by velvet-replica plating, we aim to plate approx 200–300 colonies on the SD – Leu/Arg/Lys + canavanine/thialysine medium.
12. To ensure the pins are cleaned properly and to avoid contamination in the wash procedure, the volume of wash liquids in the cleaning reservoirs is designed to cover the pins sequentially in small increments. For example, in the first step, only the tips of the pins should be submerged in water. As the pins are transferred through the cleaning reservoirs to the final ethanol step, the lower halves of the pins should be covered.
13. To reduce waiting time during the sterilization procedure, it is desirable to have three to four pinning tools such that they can be processed through the sterilization and pinning procedure in rotation.
14. To minimize contamination on the deletion mutant array (DMA), we propagate it on YEPD + G418 plates. This collection of 384-density plates should be maintained as the master plate set for SGA analysis and also as frozen stock at –80°C. The agar plates can be kept at 4°C and propagated as needed, or revived from the frozen stock once every month.
15. Pinning the query strain in the 768-format on an agar plate is advantageous as cells are evenly transferred to the subsequent mating step. One query plate should contain a sufficient amount of cells for mating with eight plates of the DMA.
16. The DMA can be reused for three to four rounds of mating reactions.
17. It is important to keep the sporulation plates at approx 22–24°C for efficient sporulation. The resultant sporulation plates can be stored at 4°C for up to 4 mo without significant loss of spore viability, and provide a source of spores for random spore analysis and tetrad analysis.
18. The barcode microarrays can be used as an alternative method to score the double mutant for fitness defects. Because each of the deletion mutants is tagged with two unique oligonucleotide barcodes, their growth rates can be monitored within a population of cells. As shown in **Fig. 3**, the steps for creating double mutants can be carried out in pooled cultures and synthetic fitness defects can be analyzed using the barcode microarrays where the hybridization intensities reflect the representation of the double-mutant meiotic progeny. A technique called synthetic lethality analysis by microarray (SLAM) uses a transformation-based strategy to create a pool of double mutants, which can then be analyzed by the barcode microarrays ***(11)***.
19. In addition to visual inspection of the double mutants, we have developed a computer-based scoring system, which generates an estimate of relative growth rates

from the area of individual colonies, as measured from digital images of the double-mutant plates. Statistical significance can be determined for each strain by comparing the measurements between the mutants and wild-type controls.

20. The programs FunSpec (http://funspec.med.utoronto.ca/) and FuncAssociate (http://llama.med.harvard.edu/cgi/func/funcassociate) are used to assign functional annotations in order to assist the sorting of putative interactions. FunSpec takes a list of genes as input and produces a summary of functional annotations from the MIPS and GO databases that are enriched in the list. FuncAssociate takes a list of genes as input and produces a ranked list of the GO annotations as enriched or depleted within the list.
21. The spores are derived from the sporulation step in the SGA procedure. Alternatively, heterozygous diploids of the query mutation and test mutation can also be generated independently by mating the *MAT*α query strain to the *MAT***a** deletion strain of interest (*xxxΔ::kanR*). The resulting diploids can then be induced for sporulation and used in the random spore analysis and tetrad analysis.
22. The expected number of *MAT***a** meiotic progeny on each medium should be roughly equal. SD – His/Arg/Lys + canavanine/thialysine allows germination of the *MAT***a** meiotic progeny that carries the *can1Δ::MFA1pr-HIS3* and *lyp1Δ* markers.
    (SD/MSG) – His/Arg/Lys + canavanine/thialysine/G418 allows the germination of the *MAT***a** meiotic progeny that carries the *can1Δ::MFA1pr-HIS3* and *lyp1Δ* markers, and the *kanR*-marked gene deletion.
    (SD/MSG) – His/Arg/Lys + canavanine/thialysine/clonNAT allows the germination of the *MAT***a** meiotic progeny that carries the *can1Δ::MFA1pr-HIS3* and *lyp1Δ* markers, and the *natR*-marked query mutation.
    (SD/MSG) – His/Arg/Lys + canavanine/thialysine/G418/clonNAT allows the germination of the *MAT***a** meiotic progeny that carries the *can1Δ::MFA1pr-HIS3* and *lyp1Δ* markers, and the double mutations of the *natR*-marked query and *kanR*-marked gene deletion.
23. Because we cannot add the antibiotics (G418 and clonNAT) into the medium for tetrad analysis, the closest conditions to the double mutant selection step is synthetic dextrose (SD/MSG) Complete medium. This medium is more sensitive than the conventional rich medium in detecting subtle growth defects.

Acknowledgments

We thank B. Garvick and L. Hartwell for suggesting the use of the *LYP1* marker; D. Burke for suggesting the use of MSG in minimal medium containing antibiotics; B. Andrews, M. Tyers, D. Burke, J. Brown, M. Ashby, J. Rine, C. Roberts, D. Shoemaker, B. Drees, and S. Fields for helpful discussions during the method development; N. Page and H. Bussey for supplying the deletion strains; S. Raghibizadeh for designing and building the robotic system for yeast cell manipulation; G. Sprague Jr. for insight into the use of a mating-type specific promoter driving a selectable marker; R. Brost and A. Parsons for helpful discussion and comments on the manuscript.

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18

Two-Dimensional Agarose Gel Analysis of DNA Replication Intermediates

Alain T. Dandjinou, Michel Larrivée, Ralf E. Wellinger, and Raymund J. Wellinger

Summary

The neutral/neutral (N/N) two-dimensional (2-D) agarose gel technique is a useful tool for understanding the mechanisms leading to the complete duplication of linear eukaryotic chromosomes. For the yeast *Saccharomyces cerevisiae*, it has been used to localize and characterize origins of replication as well as fork progression characteristics in a variety of experimental settings. The method involves running a first-dimension gel in order to separate restriction-digested replication intermediates of different mass. A gel slice containing the continuum of replicating DNA is then cut and subjected to a second-dimension gel, such as to resolve replication intermediates of varying topology. The 2-D gel is then blotted and probed to allow an examination of replication intermediates in specific DNA regions.

Key Words: DNA replication; origin activation; two-dimensional agarose gels.

1. Introduction

Eukaryotic organisms duplicate chromosomal DNA by initiating polymerization at many sites throughout the genome, called replication-initiation sites ***(1,2)***. Genetic studies can yield important insights into the requirements for origin function, but are in many instances inadequate to physically map sites of replication initiation or probe the molecular mechanisms of fork progression. Early on, techniques such as electron microscopy and autoradiography were used to address these issues, but turned out to be limited in resolution ***(3–5)***. Using knowledge gained in the analyses of the migration properties of branched and circular DNA in various types of agarose gels, in the late 1980s two groups independently introduced two-dimensional (2-D) gel electrophoresis techniques to get a higher-resolution assessment of the mechanisms of eukaryotic

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

chromosome replication ***(6,7)***. These studies were pioneered using yeast DNA and have later found applications in the analyses of DNA replication in prokaryotic and other eukaryotic organisms as well.

In this chapter, we describe the standard neutral-neutral (N/N) 2-D gel technique in detail and will refer the reader to appropriate references for a number of other agarose-gel based replicon mapping techniques (*see* **Note 1**). We also refer the reader to the work of Raghuraman et al. ***(8)*** concerning a high-throughput approach based on DNA microarray technologies, which allows monitoring of locations, times of activation, directions of replication fork, as well as fork migration rates of the most efficient origins in the yeast genome. The N/N 2-D technique may not be the appropriate approach for all situations, but when embarking on replication fork studies, it is a very good starting point. Once this technique is experimentally mastered, it becomes easier to switch to others.

When a DNA molecule is replicated, it progressively doubles in mass and it adopts various bubbled or branched topologies, depending on where the closest site of replication initiation is located (**Fig. 1**; ***9***). In the N/N 2-D gel electrophoresis method, DNA restriction fragments containing replication intermediates are separated first in an agarose gel on the basis of molecular mass (extent of replication). The effects of various molecular shapes or topologies on migration characteristics are minimized in this dimension (low agarose concentration and weak electric field). The gel conditions for the subsequent second dimension will exacerbate the effect of different topologies of DNA of the same mass (high agarose concentration, strong electric field, high ethidium bromide [EtBr] concentration). The position in the 2-D gel of replication intermediates eventually is visualized by Southern blotting and hybridization with appropriate probes.

In addition to origin identification and mapping (**Fig. 1**), the N/N 2-D gel technique can also be used to identify points of fork stalling (***10,11*** and references therein) and recombination intermediates ***(12)***. Later improvements of this N/N system involve an in-gel cleavage of the target DNA fragment by a restriction enzyme, which allows a determination of the direction of fork movement through a given DNA fragment (**Fig. 2**; ***13***).

We applied the N/N 2-D gel electrophoresis technique to determine the timing of origin activation as well as direction of fork movement for a linear plasmid ***(14)***, establish a mechanistic link between the conventional replication machinery and telomere maintenance ***(15)***, and demonstrate differential replication machinery requirements for chromosome-ends replicated by leading- vs lagging-strand synthesis ***(16)***.

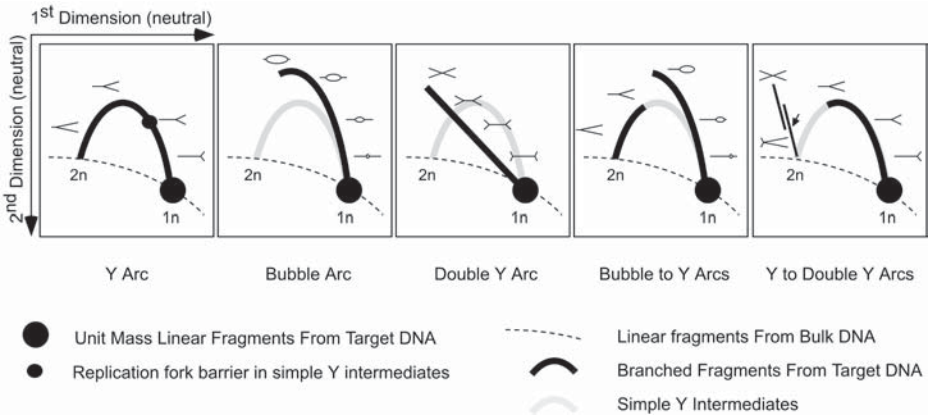

Fig. 1. Schematic representation of various N/N 2-D gel patterns. The major black oval (the "1n spot") represents the target linear restriction fragment of unit mass. This basically is the double-stranded DNA restriction fragment that is being investigated for replication intermediates. If one was to use DNA lacking any replication intermediates in the N/N 2-D technique, this would be the only signal visible. In the text, we refer to this fragment as the unit mass fragment. The migration patterns expected after probing for the replication intermediates of this unit mass fragment undergoing different modes of replication consist of arc(s) with characteristic shapes, as indicated in heavy black line. The corresponding replication intermediates are shown above the arc. The lightly shaded arcs, indicating the migration pattern of simple Y intermediates, are included for reference. The dashed arc outlines nonreplicating linear fragments from bulk DNA. The 2n spot on this arc denotes the spot of double the mass of the unit mass fragment. Simple Y intermediates containing forks stalled at a replication fork barrier are indicated by a small black oval (the RFB spot) at its corresponding position. X-shaped molecules corresponding to almost fully replicated intermediates and recombination hemicatenates migrate as nearly vertical spikes above the 2n spot. The 2n spike corresponding to hemicatenates is indicated by an arrow. Adapted with permission from **ref. *6***.

2. Materials

2.1. Detection of DNA Replication Intermediates

1. Agarose, ultrapure (USB, Cleveland, OH), or low melting temperature Agarose SeaPlaque GTG (FMC Bioproducts, Rockland, ME) for in-gel digestion.
2. 1X TBE: 90 m*M* Tris base, 90 m*M* boric acid, 2 m*M* ethylenediaminetetraacetic acid (EDTA), pH 8.0.
3. 10X DNA loading buffer: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 50% glycerol, 100 m*M* EDTA, pH 7.5.

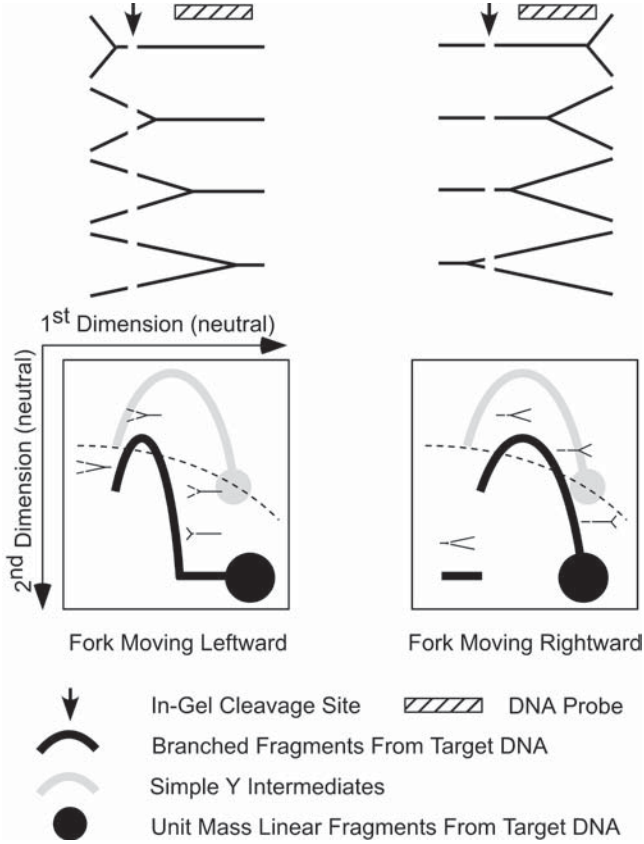

Fig. 2. Schematic representation of leftward and rightward fork direction gel pattern. In-gel cleavage of replication intermediates at an asymmetric position generates two sub-fragments. Probing for the larger sub-fragment allows discrimination between leftward and rightward fork direction. The respective characteristic arcs are indicated in heavy black line. The corresponding replication intermediates are shown above the arc. The downward arrow marks the in-gel cleavage site. The probe is represented by hatched bars. The lightly shaded arcs, which indicate the migration pattern of undigested simple Y intermediates, are included for reference. The black oval is the "1n spot" representing the unit mass fragment. The dashed arc outlines nonreplicating linear fragments from bulk DNA. Adapted with permission from **ref. *13***.

4. DNA size marker: Any commercially available size markers will work; we use the 1 kb DNA Ladder (Invitrogen, Carlsbad, CA).
5. Ethidium bromide (EtBr) 10 mg/mL: Dissolve 1 g of EtBr (USB) in 100 mL of water. Store away from light sources. CAUTION: EtBr is a powerful mutagen and is moderately toxic. Gloves should be worn at all times when handling EtBr or solutions containing it.

6. UV light source 360 nm. Caution: UV light is damaging for eyes and exposed skin. Protective eyewear and gloves should be worn at all times while using a UV light source.
7. Restriction enzymes and buffers: As detailed by the company selling the respective enzyme. We usually use restriction enzymes from New England Biolabs (NEB, Beverly, MA) with the appropriate buffer.
8. Positively charged nylon transfer membrane (Hybond-N+ membrane, Amersham Biosciences, Buckinghamshire, UK).
9. Bio-Max MS film (Eastman Kodak Company, Rochester, NY).
10. UV Stratalinker 2400 (Stratagene, La Jolla, CA).

2.2. Determination of the Direction of Fork Movement

1. Falcon tubes, 15 mL (Becton Dickinson, Franklin Lakes, NJ).
2. Heat-sealable plastic bag (Fisher, Fair Lawn, NJ).
3. Dialysis clip (Spectra/Por, Rancho Dominguez, CA).

3. Methods

3.1. Detection of DNA Replication Intermediates

Because optimal electrophoresis conditions vary depending on DNA-fragment sizes, the following protocol should be considered as a guide. The conditions given below are best suitable for replication intermediates of restriction fragments in the 1.5–7.0 kb range (*see* **Note 2**). We refer to the given DNA fragment to be analyzed as unit mass fragment (*see* **Fig. 1**).

3.1.1. DNA Preparation and Digestion

1. Grow yeast cells to early-mid exponential phase (OD_{660}: 0.4–0.6) in the appropriate medium under the desired experimental conditions (*see* **Note 3**). Harvest cells by centrifugation at 1800*g* for 10 min.
2. Isolate total genomic DNA such as to maintain the integrity of replicating DNA. DNA preparations must be clean enough to permit subsequent restriction endonuclease digestion (*see* **Note 4**).
3. If necessary, incorporate additional steps to enrich for replicating molecules (*see* **Note 5**).
4. Generate a restriction fragment of the DNA portion to be analyzed by digesting approx 5–10 µg of the isolated DNA with the appropriate restriction enzyme(s) in a total volume of 25–50 µL and transfer the appropriate amount in a clean tube (*see* **Subheading 3.1.2., step 3**). Do not heat-inactivate restriction enzyme (*see* **Note 6**).

3.1.2. DNA Fragment Separation by Agarose Gel Electrophoresis (First Dimension)

1. Prepare 500 mL of a 0.35% agarose gel in 1X TBE buffer WITHOUT EtBr. After dissolution of the agarose by heating, cool down to 55–60°C and pour onto a gel-casting

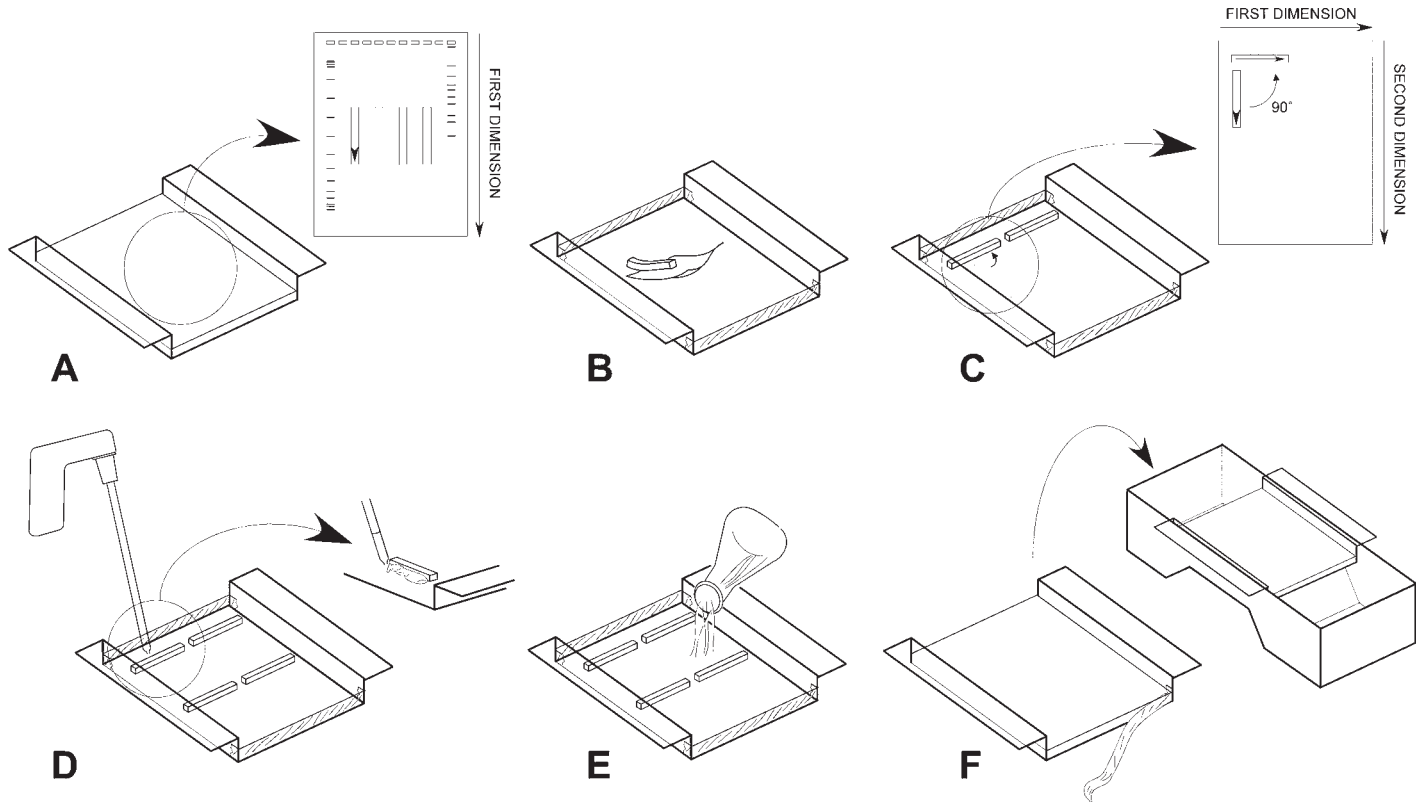

Fig. 3. Schematic representation of second dimension agarose gel pouring. *See* text describing **Subheading 3.1.2., step 7** through **Subheading 3.1.3., step 4** for details.

platform. We routinely aim for a 20 cm wide, 25 cm long, and 0.7 cm thick gel (**Fig. 3**). It contains 20 wells, each 0.6 cm wide, 0.5 cm deep, and 0.125 cm thick (*see* **Note 7**).
2. Place the gel-casting platform containing the set gel in the electrophoresis tank and add sufficient 1X TBE to cover the gel completely (buffer level about 1 mm above gel). Gently remove the comb.
3. Prepare samples and at least one size marker covering the size range of interest by addition of the appropriate amount of 10x loading buffer. We load approx 5 µg of total yeast DNA for the analysis of rDNA replication (**Fig. 4B**; *see* **Note 8**). The final DNA concentration in the loading buffer should not exceed 200 ng/µL. Apply samples to wells, leaving an empty well between each adjacent sample (*see* **Note 9**). Size markers are loaded into the outermost wells of the gel.
4. Set the voltage to 0.7 V/cm (in our system approx 15 V for a 20 cm gel) and subject the first dimension to constant voltage electrophoresis for approx 42 h at room temperature. While the first dimension is running, prepare enough running buffer (1X TBE buffer supplemented with 1 µg/mL EtBr) for the second dimension and pre-equilibrate to 4°C (*see* **Subheading 3.1.3., step 4**). Run the first dimension long enough to resolve the size marker corresponding to the unit mass fragment. In our conditions, the unit mass fragments (4.577 and 4.720 kb) are located approx 16 cm from the well (**Fig. 4B**). Change the buffer once midway through the run.
5. When the desired migration distance is reached, stop electrophoresis and stain the gel by immersing the gel platform into a 1X TBE buffer containing 0.3 µg/mL EtBr. Be careful when handling the 0.35% gel because it is very slippery and fragile. If the gel was migrated in the presence of EtBr the staining step is not necessary. While staining the first-dimension gel, prepare the second-dimension gel as described in **Subheading 3.1.3., step 1**.
6. Place a ruler along the gel as a scale guide and photograph the gel under a long-wave (360 nm) UV light source (*see* **Note 10**).
7. Estimate the position of the unit mass fragment and the position of a fragment double that size in the sample-lanes by inferring from the mobilities of the known size marker DNAs. Using a clean, sharp scalpel and a ruler to obtain a straight cutting edge, carefully cut out the lane and include 1 cm below the unit mass fragment and 3–4 cm above the position of the double of the unit mass fragment. Minimize the size of the gel slabs by removing DNA-free agarose on each side of the lane as much as possible (**Fig. 3A**; *see* **Note 11**). Slip a thin piece of flexible support (for example, a used X-ray film) under the gel slab.

3.1.3. Separation in the Second Dimension

1. Prepare 500 mL of a 1.0% agarose gel in 1X TBE buffer as above. Add EtBr to a final concentration of 0.3 µg/mL. Make sure that the agarose is well-dissolved and let equilibrate to approx 60–65°C.
2. Meanwhile, carefully transfer the excised first-dimension slabs to a clean gel platform similar to the one used for the first dimension (**Fig. 3B**). Rotate the slabs 90° from the original direction of electrophoresis so that the topmost portion

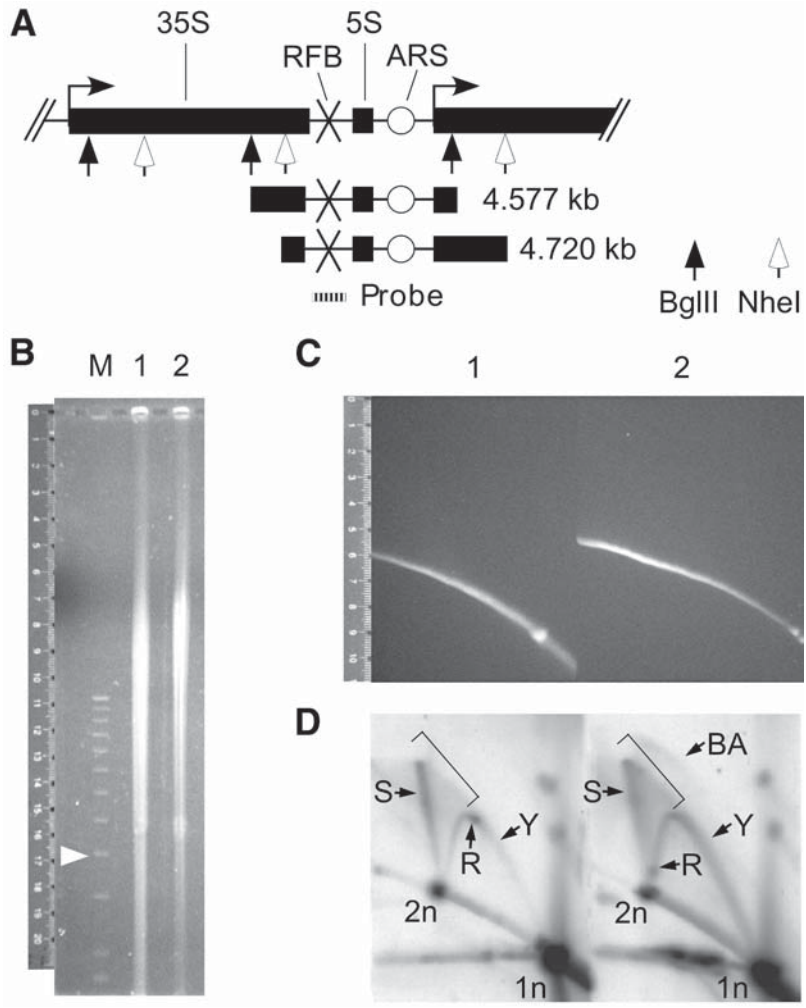

Fig. 4. Two-dimensional gel analysis replication intermediates in the yeast rDNA. **(A)** Organization of the rDNA cluster on the right arm of chromosome XII. The yeast rDNA locus comprises 100–200 repeats of a 9.1 kb transcription unit enclosing the 35S and 5S precursors, one autonomous replicating sequence (ARS) and a replication fork barrier (RFB). *Bgl*II and *Nhe*I individually cleave the rDNA locus into two fragments of 4.577*/4.560 kb and 4.720*/4.417 kb, respectively. The asterix indicates the unit mass fragments containing the ARS. **(B)** First-dimension gel. 5 µg of total yeast DNA were digested either with *Bgl*II or with *Nhe*I and the resulting fragments were separated in a 0.35% agarose gel (lane 1 and lane 2, respectively), along with a 1 kb size marker. The gel was EtBr stained, and the lanes were cut as described in the text. The arrow indicates the position of the 4 kb marker. **(C)** Second-dimension gel. Lanes

becomes the left in the second dimension (**Fig. 3C**). Our platform may accommodate up to four samples (two across the top and two in the middle; **Fig. 3D**).

3. Properly align the first dimension gel slabs with a ruler and seal them in place by pipetting a small quantity of the agarose set aside in **step 1** BEHIND the first-dimension gel slabs (**Fig. 3D**; *see* **Note 12**).
4. After the first-dimension slabs have been firmly immobilized on the platform, slowly pour the remaining of the second-dimension agarose around the first-dimension slices until covering them, and taking care not to trap air bubbles at the 0.35%/1.0% boundary (**Fig. 3E**; *see* **Note 13**). After the gel has hardened (**Fig. 3F**), place the gel-casting platform containing the set gel in the electrophoresis tank and move it into a cold room (4˚C). Add enough buffer, pre-equilibrated at **Subheading 3.1.2., step 4**. to cover the gel. Let the gel equilibrate to 4˚C for approx 30 min.
5. Set the voltage on the power supply to approx 7–8 V/cm (in our gels, this comes down to 140–160 V with an amperage of 120–180 mA) and run the second dimension until the smallest linear fragments to be analyzed have migrated about 10–12 cm. Monitor migration by visualizing under UV light. An arc indicative of bulk DNA (double-stranded linear restriction fragments of yeast chromosomal DNA) should be visible (**Fig. 4C**). An absence of this arc predicts faint replication intermediates signals. To avoid excessive heating and maintain constant EtBr concentration along the gel length, circulate the buffer such that it moves in the opposite direction from the migration of the DNA (from anode to cathode) in the gel box (*see* **Note 14**).
6. Following electrophoresis, place a ruler along the gel as a guide and photograph the gel as previously.
7. Transfer the DNA onto a nitrocellulose membrane using standard alkaline capillary Southern blotting and hybridize the membrane to a labeled probe specific for

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Fig. 4. *(continued)* 1 and 2 were excised from the first-dimension gel, embedded into a 1% agarose gel, and subjected to the second-dimension electrophoresis. The gel photograph reveals diagonal arcs generated by nonreplicating linear fragments from bulk DNA. **(D)** 2-D gel analysis of replication intermediates of rDNA. The second-dimension gel **(C)** was blotted and the replicating *Bgl*II fragment (lane 1) or *Nhe*I fragment (lane 2) were detected using a 120 bp probe encompassing the RFB region. The autoradiogram reveals characteristic replication patterns consistent with a model in which only a fraction of all origins in the rDNA cluster are functional in each cell cycle (for detailed interpretations, *see* **refs. *13*** and ***34***). Y indicates a simple Y-arc (passive replication). BA indicates a bubble arc (active ARS within the restriction fragment). R indicates the RFB. The bracket indicates fragments with a stalled fork being replicated by a second fork. S indicates the 2n-spike corresponding to recombination intermediates, including hemicatenates and Holliday junctions. Note that an origin located very close to the end of a given unit mass fragment would yield only very small bubbles. This is the case here for the *Bgl*II fragment. Such very small bubbles in general are very hard to detect and there is no detectable signal for those bubbles on the 2-D gel shown here.

the unit mass fragment. Expose to appropriate film to visualize replication intermediates according to **Fig. 1** ***(17)***. If necessary, quantitate DNA signals (*see* **Note 15**). An example of N/N 2-D gel pattern is given in **Fig. 4D**.

3.2. Determination of the Direction of Fork Movement

The passage of a single replication fork through a restriction fragment generates simple Y replication intermediates (**Fig. 2**). To gain information about the direction of the fork movement through a given unit mass fragment or on how these simple Y's are generated, an in-gel restriction cleavage step was introduced between the first and the second electrophoretic dimensions of the original N/N method (*see* **Note 16**; ***13***). Upon hybridization of a probe targeting the larger of the two restriction fragments, characteristic 2-D gel patterns are indicative of the direction of the fork movement (*see* **Note 17**). This modification is also useful to quantitate the frequency of origin usage ***(13,18)***.

1. Prepare the first-dimension gel with low-melting agarose (SeaPlaque, GTG) instead of standard agarose (*see* **Note 18**) and perform first-dimension electrophoresis as described in **Subheading 3.1.2., step 1**.
2. Excise the first-dimension gel as described in **Subheading 3.1.2., step 7**. Carefully slide into a 15-mL Falcon tube and fill to overflowing with the appropriate restriction buffer. Incubate at room temperature for 6 h, with gentle agitation. Change the buffer once midway through the incubation. Meanwhile, cut and seal a heat-sealable plastic bag such that it has the shape of a tube 2–3 cm longer than the gel slab and just narrow enough to keep the gel slab in constant contact with the buffer. Seal one end of the bag using a dialysis clip.
3. Drain the restriction buffer from the Falcon tube and, holding the neck of the bag, allow the gel slab to sink to the bottom of the bag. Fill the bag with fresh restriction buffer supplemented with restriction enzyme (1.5 U of restriction enzyme per µL of buffer; *see* **Notes 16, 17,** and **19**). Clip the bag just above the gel slab, avoiding trapping air bubbles. Incubate at the appropriate temperature for 6 h (*see* **Note 5**) with gentle agitation.
4. Proceed with the second dimension as described in **Subheading 3.1.3.**, taking care to adapt reaction conditions to the smaller size of target fragments generated.

4. Notes

1. There are additional agarose-gel-based replicon mapping techniques that one may consider when studying the mechanics of DNA replication:
   a. 1-D gel electrophoresis: two methods have been developed in order to map replication fork stalling points ***(19)***. Both techniques can be useful for rapid identification of an active replication fork in a given DNA fragment by using only one dimensional gel electrophoresis. The first approach consists of a limited digestion of purified genomic DNA with Mung Bean nuclease, a single-strand-specific nuclease, in order to release replication intermediates from bulk genomic DNA. The replication intermediates derived of replica-

tion bubbles are separated from the unreplicated genomic DNA by neutral agarose gel electrophoresis. The sequence of interest is visualized by Southern blotting with hybridization to specific DNA probe. The second approach is also a fast procedure to visualize a replicating origin. Undigested genomic DNA is analyzed by using denaturing one-dimensional agarose gel electrophoresis, in which replication intermediates are released from parental DNA as small fragments. Because parental DNA is much larger, it is easily resolved from the replication intermediates. The replication intermediates are detected with specific DNA probes after Southern blotting.

b. Neutral/Alkaline (N/A) 2-D gel electrophoresis: the direction of replication fork movement can be directly determined by the N/A 2-D gel electrophoresis, firstly adapted for yeast in Huberman's lab ***(7,20)***. The first-dimension gel is essentially the same as discussed above for the N/N 2-D technique. However, the second dimension is carried out in an alkaline buffer. Thus, the second dimension conditions allow the separation of nascent DNA strands of various sizes from parental DNA strands, resulting in a characteristic arc pattern on the subsequent Southern blot. For example, the N/A 2-D replicon-mapping technique detects an origin of replication by hybridizing different probes distributed along the length of the restriction fragment analyzed. This hybridization procedure will also yield information about the direction of fork progression through a given DNA fragment and facilitate the detection of points of replication termination. The N/N and N/A 2-D gel electrophoresis techniques provide complementary information, both having their strengths and limitations (***7,13***).

c. Three-dimensional gel electrophoresis: standard N/N 2-D gel electrophoresis can be further analyzed by performing a third dimension as originally described by Liang and Gerbi ***(21)***. Briefly, vertical gel slices (perpendicular to the first dimension) are cut out from the two-dimensional slab and each one is rotated 90° and placed for a third-dimension run. Each gel slice should carry bulk DNA, forks and/or bubbles, previously resolved with the second dimension. The DNA is then subjected to an alkaline gel electrophoresis for the third dimension. Under these conditions, denatured DNA is resolved on the basis of molecular mass, allowing the separation of nascent strands from parental strands. This technique is useful for determining the mass of forks and bubbles, the presence of broken bubbles as well as for analyzing the initiation region of replication ***(21,22)***.

2. When analyzing genomic DNA fragments, it is a good idea to establish experimentally that this fragment is indeed generated by the used restriction enzymes prior to embarking on N/N 2-D analyses. The method may also accommodate fragments of smaller (1.0 kb) or larger sizes (up to 20 kb), provided that the electrophoretic conditions are altered on the basis of pilot experiments with fragments of known size and replication patterns. For smaller fragments, the first dimension is typically run at a slightly higher agarose concentration (0.6–0.7%), whereas the second dimension is run in a 2% agarose gel ***(9)***. For larger frag-

ments, lower agarose concentrations and lower voltages in both dimensions are required ***(23,24)***.

3. Because the time of actual replication of any given DNA fragment in the size-range is very short, replication intermediates are generally very rare. Therefore, it is critical to enrich cells in S-phase using alpha-factor synchronization or elutriation ***(25)***.
4. Work rapidly and keep samples cold whenever possible. Branched intermediates are fragile. Shearing, nicking, and nascent strand extrusion (removal of nascent DNA at the fork followed by rewinding of parental strands) must be minimized because they may lead to loss of signal as well as generation of artifacts ***(9,26)***. Mechanical shearing can be minimized by use of large-bore pipet tips and gentle hand mixing. Nascent-strand extrusion may be reduced by avoiding low ionic strength and excessive heat (e.g., during restriction enzyme digestion and subsequent inactivation of the enzymes; *see* **Note 5**). For DNA preparations, CsCl is simply the best method ***(17)***; next comes Qiagen columns and standard purification methods that very much depend on the handling. Additional established techniques can be found ***(7,14,27–30)***.
5. Even if enrichment methods have been previously used to increase the proportion of S-phase cells, additional methods of enrichment for replicating molecules may be helpful to increase the signal. These methods include isolation of nuclear matrix (replication forks are attached to the nuclear matrix; ***31,32***), or affinity purification of DNA with single-strand regions via BND-cellulose ***(7)***. These procedures may be used alone or in combination to produce DNA preparations that are further enriched in replication intermediates.
6. Carefully choose the restriction enzyme(s) for this initial digest to generate a fragment of 3.0–6.0 kb. The restriction enzyme(s) should cleave the DNA to completion without star activity or degradation. Restriction enzyme digestions are usually performed at elevated temperatures and low ionic strength, conditions that favor nascent strand extrusion (*see* **Note 3**). It is therefore important to incubate DNA for the shortest time necessary to obtain maximum cleavage. Moreover, some commercial restriction enzymes may contain nonspecific single-strand nuclease contaminants, which will destroy branched molecules and reduce signal. Verify with manufacturer's analysis sheet or test enzyme batches by incubating with single-stranded circular DNA (e.g., M13 DNA) and assay for conversion to linear form or degradation by agarose gel electrophoresis. Avoid using spermidine in the digestion buffer because it may affect DNA mobility during electrophoresis ***(26)***.
7. Huberman's lab uses TAE buffer containing EtBr at low concentration (0.1 µg/mL). TBE provides a better resolution for smaller molecules, whereas TAE provides a better resolution for larger molecules. The use of EtBr at low concentration during the first dimension does not significantly alter the mobility or integrity of replicating molecules and allows the monitoring of the progress of electrophoresis, avoiding the need to stain the gel between the first and the second dimension. The size of the gel wells results in a long, narrow gel lane with very

tight bands. The gel wells will accommodate samples of 10–30 µL and their capacity may be raised by increasing gel thickness up to 10 mm.

8. When analyzing single-copy DNA sequences and using methods to enrich either for S-phase cells or replication intermediates, we obtain good signals using 1–5 µg of DNA. Without any enrichment methods, 5–15 µg of DNA usually provide satisfactory signals.
9. Before removing the comb place the gel for 30 min at 4°C. This will make the gel more solid and maintain the slots intact. Carefully rinse the slots using a 50-mL syringe before loading the samples. This will reduce uneven distribution of DNA in the lane (a kind of shadow effect). We have noticed that samples may diffuse laterally quite a bit; thus it is advisable to separate them by at least one lane to avoid cross-contamination. To get repeatable running conditions, note the distance of the xylene cyanol and bromophenol blue dyes from the slot.
10. If the gel was migrated in presence of EtBr (Huberman method; *see* **Note 7**), the migration distance can be monitored by visualizing under UV light. However, avoid excessive exposure to UV light, as exposure to it can lead to DNA nicking.
11. If the tool used to cut the gel is not sharp, rough edges alongside the cut lanes will hamper the "stacking effect" required to form a sharp band of fragments when they enter the second-dimension gel.
12. The slab's edges should be perfectly straight to ensure that the 0.35%/1.0% interface forms a perfectly horizontal line that will produce a sharp second-dimension band with no distortions. For small fragments, the agarose gels are more concentrated in both dimensions (*see* **Note 2**). Because the 2% agarose in the second dimension is not easily poured at 55°C and to avoid exposing the DNA to high temperatures (*see* **Notes 3** and **5**), the second-dimension agarose may be poured before inserting the first-dimension slabs ***(9)***.
13. Use a pipet tip to remove any bubbles that may be trapped at the 0.35%/1.0% interface (*see* **Note 12**).
14. Verify the amperage, it should not exceed 200 mA otherwise the gel will melt. Excessive heating may also be avoided by circulating the buffer. If a refrigerating circulator is not available, remove the buffer every hour and replace with fresh pre-equilibrated buffer.
15. After blotting, the nylon membrane is rinsed, air-dried, and the DNA is cross-linked by UV irradiation at 120 mJ for approx 30 s (auto cross-link setting on the irradiator). We use either DNA probes labeled to high specificity and that were generated by random priming or PCR labeled fragments. Exposure time may vary from a few hours to a few days depending on the abundance of the target fragment. For rough measurements, DNA amounts may be measured directly from the gel photograph or autoradiogram. For more precise work, quantify each signal using PhosphorImaging.
16. The in-gel digestion site should be located one-quarter to one-half of the way from one end of the given unit mass fragment to be analyzed. For this analysis, it is preferable that there is no actual site of initiation on the fragment, because such bubble intermediates could complicate the interpretation.

17. Although the smaller fragment could also be probed, replication intermediates from such small fragments are extremely difficult to detect ***(33)***.
18. We found that in-gel restriction enzyme digestion of DNA is more efficient in low-melting agarose.
19. The enzyme for the in-gel digestion should be compatible with the agarose such as to cleave the embedded DNA to completion or near completion (*see* **Note 5**).

Acknowledgments

We thank all past and present members of the Wellinger lab, specifically J. Parenteau, for discussions and help with the applications of some of the techniques described here. Research in our laboratory is supported by grants of the Canadian Institutes of Health Research (CIHR) and the Canadian Cancer Society (NCIC). ML was supported by an MRC studentship. RJW is a chercheur-National supported by the Fonds de la Recherche en Santé du Québec (FRSQ). REW is a Ramon y Cajal research fellow (2003-20-10-542M-750) supported by grants of the Ministerio de Ciencia y Tecnolgia, Spain (BIO2003-07172).

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Chromatin Assembly in a Crude Fraction From Yeast Cells

Karen M. Robinson and Michael C. Schultz

Summary

The mechanisms of biological chromatin assembly and their regulation have been studied intensively using cellular extracts, particularly those from the embryonic cells of various metazoans. Here we describe how to prepare and use a crude chromatographic fraction from budding yeast, which also supports biological chromatin assembly. In this system, nucleosomes are assembled by a replication-independent mechanism into physiologically spaced arrays that significantly protect underlying DNA from restriction endonuclease digestion. The formation of correctly spaced nucleosome arrays absolutely requires ATP and exogenous core histones of yeast or *Drosophila*. We have explored how cell cycle and DNA damage signals affect assembly activity in this system.

Key Words: Chromatin assembly; nucleosome; histone; yeast; cell cycle; DNA damage; micrococcal nuclease digestion; plasmid supercoiling; restriction endonuclease accessibility.

1. Introduction

At its most fundamental level, chromatin assembly is the deposition of core histones on DNA to form nucleosomes. The process requires chromatin assembly factors that function in two assembly pathways, one that is coupled to DNA replication and one that is not ***(1,2)***. "Replication-dependent" chromatin assembly is important for histone deposition on newly synthesized DNA. "Replication-independent" assembly may back up the replication-coupled pathway in dividing cells and replace nucleosomes that are spontaneously lost outside of S phase (including in terminally differentiated cells). Recent evidence also suggests that nucleosome displacement by RNA polymerases is counteracted mostly by replication-independent assembly ***(3,4)***.

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

Crude extracts of *Drosophila* and *Xenopus* embryonic cells that support nucleosome reconstitution have been widely used to study the mechanisms and regulation of chromatin assembly ***(5)***. We have developed two crude replication-independent assembly systems for budding yeast, anticipating similar uses complemented by a combined biochemical and genetic approach not easily exploited in metazoans. One system uses whole cell extract ***(6)***, the other a crude fraction of yeast prepared by chromatography of whole cell or nuclear extract on a diethylaminoethyl (DEAE) resin ***(7)***. The latter preparation is referred to as the "crude DEAE" (CD) fraction ***(7)***. These ATP-dependent systems assemble nucleosomes incorporating approx 165 bp of DNA as observed in vivo ***(8)***. Whole cell extract assembles only modest nucleosome arrays using histones present in the extract. The CD fraction has two distinct advantages. First, it assembles extensive arrays of nucleosomes (as typical of assembly extracts from metazoan cells). Second, the investigator has control of the histone composition of the assembly reaction because histones are added to reconstitute assembly. The present report describes preparation of the CD fraction and its use in biochemical studies of chromatin assembly.

Our previous work has revealed that assembly in the CD fraction is partly dependent on Asf1, a chaperone that binds to histones H3 and H4 ***(7)***. Asf1 is also known to function in pathways of replication-coupled chromatin assembly that are induced during S phase and in response to DNA damage ***(1,2)***. However, it is not clear if replication-independent assembly involving Asf1 is responsive to cell cycle or DNA damage cues. We have examined this possibility using the CD fraction.

2. Materials

2.1. Cell Culture

1. YPD1%AS: 1% yeast extract, 2% bacto-peptone, 2% glucose, 1% $(NH_4)_2SO_4$. If necessary, the medium should be adjusted to pH 6.5 with sodium hydroxide.

2.2. Spheroplasting

See **Note 1** for a discussion of the protease problem. Labile components such as protease inhibitors and reducing agent are added to spheroplasting and other buffers just prior to use with stirring.

1. Individual protease inhibitor stocks. Phenylmethyl sulfonyl fluoride (PMSF), 0.2 *M* in isopropanol; store at room temperature. Benzamidine-HCl, 1 *M* in water; store at –20°C in small aliquots. 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 0.2 *M* in water; store at –20°C. Sodium bisulfite stock (a protease inhibitor); 0.95 g/10 mL water; prepared just before use (*see* **Note 2**).
2. 2 *M* Tris-HCl, pH 8.0.

3. S buffer. 1.1 *M* sorbital, 1% yeast extract, 1% $(NH_4)_2SO_4$, 2% bactopeptone, 2% glucose, 40 m*M* Tris-HCl, pH 7.5. Immediately before use, add to final concentration 1 m*M* PMSF, 2 m*M* benzamidine, 2 m*M* sodium bisulfite, 10 m*M* β-mercaptoethanol.
4. Wash buffer. 1.1 *M* sorbital, 10 m*M* Tris-HCl, pH 6.8, 2 m*M* ethylenediaminetetraacetic acid (EDTA). Immediately before use add to final concentration 1 m*M* PMSF, 2 m*M* benzamidine, 2 m*M* sodium bisulfite.
5. Spheroplasting enzyme. We use β-endoglucanase prepared as outlined in **ref. *9***, at a concentration of 16000 U/g cells. Zymolyase™-100T (Seikagaku America, East Falmouth, MA) is also suitable; detailed instructions for the use of Zymolyase are presented in **ref. *10***. The amount of either enzyme used depends on the yeast strain and should be determined empirically.

2.3. Preparation of 190,000g Supernatant (S-190)

1. Dimethyl sulfoxide Protease Inhibitor Cocktail (DMSO PIC), 1000X: 1 *M* PMSF, 5 mg/mL pepstatin A, 25 mg/mL *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 2.5 mg/mL chymostatin. Dissolve in DMSO and store at –20°C.
2. Aqueous PIC, 1000X: 10 mg/mL aprotinin, 5 mg/mL leupeptin, 1 *M* *p*-aminobenzamidine (4-aminobenzamidine dihydrochloride), 1 *M* ε-amino-n-caproic acid. Dissolve in water and store at –20°C.
3. β-glycero-phosphate (a general phosphatase inhibitor). 1 *M* in water; store at –20°C.
4. Buffer A: 25 m*M* HEPES-KOH, pH 7.5, 0.35 *M* NaCl, 1.5 m*M* magnesium acetate, 0.5 m*M* ethylene glycol-*bis*(2-aminoethyl)-*N,N,N′,N′*-tetracetic acid (EGTA), 10% glycerol. Make 100 mL at a time and store at 4°C. Immediately before use, add reducing agent and protease inhibitors to the following final concentrations: 0.2 m*M* AEBSF, 3 m*M* benzamidine, 3 m*M* dithiothreitol (DTT; from 1 *M* stock in water). Store at –20°C), 5 m*M* β-glycero-phosphate, 2 m*M* sodium bisulfite, plus 1X DMSO PIC and 1X aqueous PIC.
5. HEMG: 20 m*M* HEPES, pH 7.5, 0.5 m*M* EGTA, 1.5 m*M* magnesium acetate, 10% glycerol. Immediately before use, add to final concentration 2 m*M* DTT, 2 m*M* benzamidine, 2 m*M* sodium bisulfite, 1 m*M* PMSF, 1 m*M* β-glycero-phosphate. Make 1 L at a time and store at 4°C.
6. 2 *M* NaCl-HEMG: HEMG with 2 *M* NaCl. Make 1 L at a time. HEMG buffers ranging between 0 and 0.4 *M* NaCl are used during preparation of the CD fraction (*see* **Subheading 3.5.**). HEMG buffers, which include NaCl, are most easily prepared by appropriate mixing of HEMG and 2 *M* NaCl-HEMG. Store at 4°C.
7. Dounce homogenizer, 40 mL, with a tight pestle (Wheaton).

2.4. Preparation of Nuclei

1. Lysis Buffer: 18% Ficoll 400, 80 m*M* KH_2PO_4, pH 6.8, 0.25 m*M* EDTA, 0.25 m*M* EGTA. Immediately before use, add to final concentration 0.2 m*M* AEBSF, 3 m*M* benzamidine, 3 m*M* DTT, 2 m*M* sodium bisulfite, 5 m*M* β-glycero-phosphate, plus 1X DMSO PIC and 1X aqueous PIC. Make 500 mL at a time; store at 4°C.

2.5. Preparation of CD Fraction

1. yR buffer: 10 m*M* HEPES, pH 7.5, 10 m*M* potassium acetate, 1.5 m*M* magnesium acetate, 0.5 m*M* EGTA, 10% glycerol, 2 m*M* DTT, 2 m*M* benzamidine, 2 m*M* sodium bisulfite, 1 m*M* PMSF, 1 m*M* β-glycero-phosphate. This buffer can be made as a 5X stock (5 L) and diluted as needed.

2.6. The Assembly Reaction

1. yR buffer; *see* **Subheading 2.5., item 1**.
2. ATP regenerating system stocks: Stock creatine phosphate/ATP mix: 2.27 mL water, 4.25 mL of 0.5 *M* creatine phosphate, 4.25 mL of 0.5 *M* ATP, 2.97 mL of 0.1 *M* $MgCl_2$ (individual components in water). Freeze at –20°C in aliquots. Stock creatine kinase: 5 mg/mL in 10 m*M* potassium phosphate, pH 7.0, 50 m*M* NaCl, 50% glycerol. Freeze at –20°C in aliquots.
3. Complete ATP regenerating system: Immediately before use, prepare the complete ATP regenerating system by adding stock creatine kinase to stock creatine phosphate/ATP mix at a ratio of 0.84 mL creatine kinase to 99.16 mL creatine phosphate/ATP mix (the volumes should be scaled to the amount of complete ATP regenerating system sufficient for the reactions being performed). (*See* **Note 3**.)
4. Core histone storage buffer: 10 m*M* HEPES, pH 7.5, 1 m*M* EDTA, 10 m*M* potassium acetate, 10% glycerol, 1 m*M* DTT, 2 m*M* benzamidine, 2 m*M* sodium bisulfite, 1 m*M* PMSF, 1 m*M* AEBSF, 0.01% Nonidet P40 (BDH reagent, VWR International, Poole, UK), and 1X aqueous PIC.

2.7. Assay of the CD Fraction

2.7.1. Plasmid Supercoiling

1. Assembly stop solution: 20 m*M* EDTA, 0.2 *M* NaCl, 1% sodium dodecyl sulfate (SDS), 0.25 mg/mL RNase A.
2. Assembly stop solution + PK: Assembly stop solution plus 0.125 µg/µL Proteinase K (Invitrogen, Carlsbad, CA) freshly added.
3. 5X TG loading buffer: 50% glycerol, 5 m*M* EDTA, 0.1% bromophenol blue.
4. 5X Tris-Borate/EDTA (TBE) buffer (Per L): 54 g Tris base, 27.5 g boric acid, 20 mL 0.5 *M* EDTA, pH 8.0.

2.7.2. Micrococcal Nuclease Digestion

1. Micrococcal nuclease (Sigma, cat. no. N5386) is from Sigma-Aldrich (St. Louis, MO). Dissolve in 5 m*M* sodium phosphate, pH 7.0, 2.5 µ*M* $CaCl_2$ to obtain a stock of 500 U/mL.
2. Micrococcal nuclease stop solution: Mix 144 µL TE buffer (10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA), 96 µL 0.5 *M* EDTA, and 10 µL 10 mg/mL RNase A.

2.7.3. Restriction Endonuclease Accessibility

1. RNG buffer: 10 m*M* HEPES, pH 7.5, 10 m*M* KCl, 12 m*M* $MgCl_2$.

2. RNase: 10 mg/mL RNase A (Sigma, cat. no. R5000). Make in 10 m*M* sodium acetate (pH 5.2) because RNase A precipitates at high concentration and neutral pH. Boil 15 min (inactivates DNase) and allow to cool slowly to room temperature. Neutralize by adding 0.1 volumes of 1.0 *M* Tris-HCl, pH 7.4. Store at –20°C.

3. Methods

3.1. Cell Culture

In this chapter, we refer to cultures grown at 30°C. Culture at 30°C may not be suitable for all strains and should be adjusted accordingly (e.g., when using temperature-sensitive mutants).

Standard small-scale preparations of the CD fraction start with cells harvested from two 1-L cultures grown to approx 1×10^8 cells/mL in YPD1%AS (about 17 g wet cells). Large-scale preparations are obtained from 6 L of similarly grown cells (wet cell pellet of approx 50 g).

1. Grow a starter culture overnight in YPD1%AS to obtain a population of cells in late log. Seed these cells into Fernbach flasks containing 1 L each of YPD1%AS so that the culture will be in mid-log the next morning. Grow overnight.
2. Harvest the cultures at approx 3.8×10^7 cells/mL (mid log) by centrifugation in 1-L bottles.
3. Resuspend cells in 250 mL fresh, pre-warmed YPD1%AS per L of starting culture and pool to have 500 mL in each Fernbach flask. The volume of pooled cells will be 0.5 and 1.5 L for small- and large-scale preparations, respectively.
4. Grow for an additional 90 min. (*See* **Note 4**.)

3.2. Spheroplasting

1. Add Tris-HCl, pH 8.0, to 0.1 *M* (50 mL of 2 *M* stock/L) and β-mercaptoethanol to 65 m*M* (4.55 mL of 14.3 *M* stock/L) and mix at room temperature for 15 min using a stir bar.
2. Harvest the cells in a pre-weighed centrifuge bottle and determine the weight of the wet cell pellet.
3. Resuspend in 3 mL of S buffer/g cells. Remember that inhibitors and β-mercaptoethanol are added to the buffer immediately before use.
4. Add an amount of spheroplasting enzyme that will suitably digest the cells in 25–30 min. Digestion is performed with shaking, at 30°C for wild-type cells.
   Spheroplasting is monitored as follows. Dilute undigested cells in 1 mL distilled water to obtain an OD_{600} approx 0.3. Mix vigorously and record OD_{600} (the mixture will look cloudy). During spheroplasting, take samples, dilute in water as above, mix, and measure OD_{600}. The cells are spheroplasted when the A_{600} has declined to approx 20% of the starting value (i.e., ~0.06; the diluted cell mix will be clear). (*See* **Note 5**.)
5. Transfer spheroplasts to pre-weighed centrifuge bottles and spin 8 min at 3000*g*.

6. All subsequent steps are performed at 4°C using ice-cold buffers.
7. Wash pellet twice in 5 mL/g wash buffer. First wash: spin at 3000*g* for 10 min. Second wash: spin at 3000*g* for 14 min. The spin times are increased because the spheroplast pellet is very loose. It is important to resuspend the pellets gently. If necessary, use a spatula or policeman; do not vortex.
8. Determine the wet weight of the spheroplast pellet.

3.3. Preparation of S-190 From Spheroplasts

3.3.1. Spheroplast Homogenization and Preparation of S-190

1. In 40 mL Dounce homogenizer with a tight pestle (Wheaton Science Products, Millville, NJ) resuspend the pellet at 2 mL/g of spheroplasts in buffer A.
2. Break open cells using 90 strokes (30 × 3, 2-min break between each set of 30 strokes) of homogenizer. The spheroplasts for a small-scale preparation can be broken in a single homogenizer. For large-scale preparations, the spheroplast suspension is split in two for disruption in two homogenizers. Breakage is performed with the homogenizer kept in ice.
3. Incubate on ice for a total of 25 min, t_0 being the time at which homogenization started. Measure the volume of the homogenate.
4. Supplement with 1 *M* magnesium acetate so as to add 5.5 m*M* to the 1.5 m*M* already present in buffer A.
5. Spin for 2 h 10 min at 190,000*g* (400,00 rpm in SW41 rotor or 45,000 rpm in SW55 rotor; Beckman Coulter, Fullerton, CA).
6. Puncture the side of the tube with a needle and collect the supernatant into a syringe, avoiding pellet and fat layer.
7. The S-190 can be frozen in liquid nitrogen at this stage and stored at –80°C. Otherwise, proceed to dialysis.

3.3.2. Dialysis

1. Dialyze against HEMG buffer supplemented with an additional 5.5 m*M* magnesium acetate (final magnesium acetate concentration is 7 m*M*) in 6000–8000 molecular-weight, cut-off, 23-mm flat-width dialysis tubing. Use two 500-mL changes of buffer for dialysis (change buffer after 1 h and continue dialysis until conductivity is equal to that of 0.1 *M* NaCl-HEMG, usually about 45 min). Note that this dialysis does not go to equilibrium.
2. Measure the volume of the dialyzed S-190. Freeze in liquid nitrogen after reserving a 20-µL aliquot for protein determination. Store at –80°C.

3.4. Preparation of S-190 From Nuclei

3.4.1. Preparation of Nuclei

1. Prepare enough lysis buffer to resuspend spheroplasts at 2 mL buffer/g of spheroplasts. Reserve 2–3 mL of the buffer (used in **step 3** to collect homogenate that does not quickly drain from the homogenizer). Use the remainder for resuspension of spheroplasts.

2. Resuspend and break the spheroplasts on ice in a Dounce homogenizer by 40 strokes (2 × 20) with a loose pestle followed by 30 strokes (2 × 15) with a tight pestle. Each set of stokes is followed by a 2-min break. (*See* **Note 6**.)
3. Drain the homogenate into centrifuge tubes, then add the reserved lysis buffer to the homogenizer and collect the remaining homogenate. (*See* **Note 7**.)
4. Spin the lysate for 7 min at 3000*g*.
5. Collect the supernatant using a pipetter and centrifuge in a preweighed tube at 21,000*g* for 30 min.
6. Discard the supernatant and determine the wet weight of the nuclear pellet.

3.4.2. Extraction of Nuclei and Preparation of S-190

1. Transfer and gently resuspend the nuclear pellet in 3 mL buffer A/g of nuclei in a Dounce homogenizer.
2. Homogenize the nuclear suspension occasionally during a 10-min incubation on ice. Use a loose pestle.
3. Measure the volume of the homogenate, and supplement with 1 *M* magnesium acetate so as to add 5.5 m*M* to the 1.5 m*M* already present in buffer A.
4. Spin for 2 h 10 min at 190,000*g* (40,000 rpm in Beckman SW41 rotor or 45,000 rpm in SW55 rotor).
5. Use tube puncture with a needle to collect the supernatant into a syringe, avoiding the pellet.
6. Dialysis is performed as in **Subheading 3.3.2.**, or the S-190 is frozen in liquid nitrogen.

3.5. Preparation of CD Fraction

The CD fraction is prepared from dialyzed S-190, or from previously frozen but undialyzed S-190 (*see* **Note 8**). In the latter case, S-190 is thawed immediately prior to chromatography and diluted with HEMG until its conductivity is equal to that of 0.1 *M* NaCl-HEMG.

1. S-190 is chromatographed at a ratio of 40 mg protein/mL resin for nuclear extract and 50 mg/mL for spheroplast extract.
2. Apply S-190 to a DEAE-Sepharose fast flow (Amersham Biosciences, Piscataway, NJ) column that has been pre-equilibrated with 0.1 *M* NaCl-HEMG. After loading the column, wash with 6 column volumes of 0.1 *M* NaCl-HEMG.
3. Step-elute the assembly-competent fraction with 5 column volumes of 0.4 *M* NaCl-HEMG. Collect and pool only the peak fractions (to obtain 1.5–2 column volumes).
4. Transfer the CD fraction to dialysis tubing with a molecular-weight cut-off of 6000–8000 and dialyze against 500 mL yR buffer for 2 × 1 h, and 1 × 2 h. Measure conductivity to ensure that dialysis has gone to completion.
5. Freeze aliquots of the dialyzed CD fraction in liquid nitrogen and store at –80°C.

3.6. The Assembly Reaction

Chromatin assembly is performed for 1–3 h at 30°C (*see* **Note 9**). Because assembly in this system is ATP-dependent, reactions include ATP and an ATP regenerating system. The following protocol is for a "standard" reaction in a total volume of 100 µL, using CD fraction with a concentration of 4 mg protein/mL. The final composition of the reaction is: 1 mg/mL CD fraction, 7.5 µg/mL core histones, 6 m*M* $MgCl_2$, 5 µg of plasmid DNA (pGIE-0; **ref. *11***), 3 m*M* ATP, 30 m*M* creatine phosphate, 6 µg creatine kinase. The assembly reaction is assembled as follows, with mixing by gentle vortexing following each addition (note that the volumes can be scaled up to allow for multiple assays of the assembly products).

1. To 57 µL of yR buffer, add 2 µL of 0.1 *M* $MgCl_2$.
2. Add 25 µL of 4 mg/mL CD fraction protein (in yR buffer).
3. Add 1 µL of 0.75 µg/µL fly core histones (in core histone storage buffer). Incubate 15 min at room temperature.
4. Add 14.5 µL of complete ATP regenerating system (made fresh as in **Subheading 2.6., item 3**).
5. Add 1 µL of 0.5 µg/µL plasmid DNA (in TE). If supercoiling is to be analyzed (*see* **Subheading 3.7.1.**), then relaxed, closed circular plasmid is added to the reaction. This template is prepared by incubation of plasmid DNA with a DNA topoisomerase (*see* **Note 10**).
6. Allow assembly to proceed at 30°C.

3.7. Assay of the CD Fraction

3.7.1. Plasmid Supercoiling

1. To 50 µL of assembly reaction mix, add 100 µL assembly stop solution + PK. Incubate 20 min at 37ºC.
2. Extract with 150 µL phenol:chloroform. Precipitate DNA with 15 µL 2.5 *M* ammonium acetate, 340 µL 100% ethanol.
3. Dry pellets and resuspend in 5 µL TE plus 0.1 mg/mL RNAase A. Incubate 1 h at room temperature.
4. Add 1 µL 5X TG loading buffer.
5. Run the sample on a 1% agarose gel in 1X TBE buffer. The sample can be stored at –20ºC at this step for later analysis by agarose gel electrophoresis.
6. Stain gel with 0.75 µg/mL ethidium bromide (EtBr) for 20 min. De-stain in water for 20 min. An example of the expected result is shown in **Fig. 1**.

3.7.2. Micrococcal Nuclease Digestion

1. Make up micrococcal nuclease dilution series in yR buffer. Three dilutions of the stock enzyme are used: 1/15, 1/45, and 1/135 (these dilutions are 33.3, 11.1, and 3.7 U/mL, respectively).

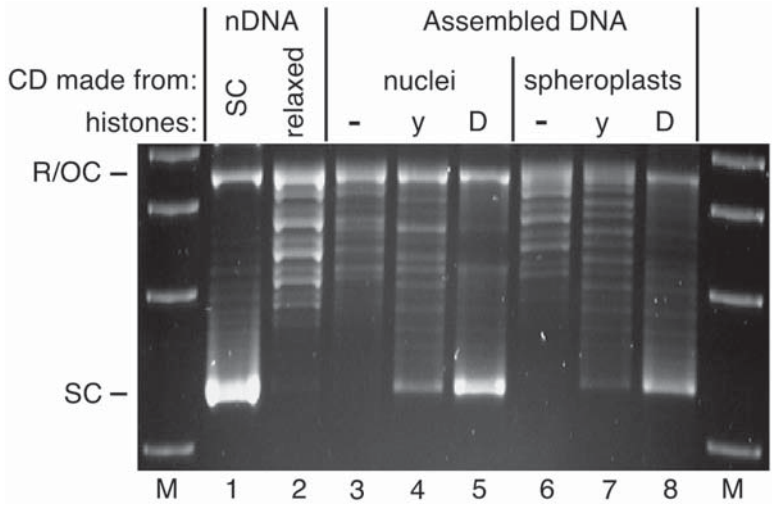

Fig. 1. Plasmid supercoiling assay of chromatin assembly supported by CD fraction from a wild-type yeast strain. Assembly reactions were done with CD fractions made from nuclei (lanes 3–5) or spheroplasts (lanes 6–8) without any histones added (lanes 3 and 6), with yeast histones added (y; lanes 4 and 7) or with *Drosophila* histones added (D; lanes 5 and 8). Plasmid DNA that was untreated (supercoiled; SC, lane 1) or relaxed with topoisomerase I (lane 2) is shown. M, 1 kbp plus ladder, Stratagene.

2. Make the assembly reaction 3 m*M* $CaCl_2$ by adding 0.1 *M* $CaCl_2$ and immediately mix by gentle vortexing. Proceed to **step 3** as quickly as possible.
3. Take 50-µL aliquots from the assembly reaction/$CaCl_2$ mix. Each aliquot will be one data point in the micrococcal nuclease titration series.
4. To each 50-µL aliquot, add 5 µL of the required dilution of micrococcal nuclease. Process the aliquots at 15-s intervals, mixing with gentle flicking after each addition of nuclease. Incubate for a total of 10 min at room temperature.
5. Stop the digestion by adding 5 µL of micrococcal nuclease stop solution; mix with gentle vortexing. Incubate for 20 min at 37ºC.
6. Add 101 µL of assembly stop solution + PK. Incubate at 37ºC for 20 min.
7. Extract with 150 µL phenol:chloroform, precipitate DNA with 15 µL 2.5 *M* ammonium acetate, 340 µL 100% ethanol.
8. Resuspend dry pellets in 5 µL TE plus 0.1 mg/mL RNase A. Incubate 1 h at room temperature.
9. Add 1 µL 5X TG loading buffer.
10. Run on a 1.25% agarose gel in 0.5X TBE buffer.
11. Stain gel with 0.75 µg/mL EtBr for 20 min. De-stain in water for 20 min. An example of the expected result is shown in **Fig. 2**. Plasmid supercoiling and

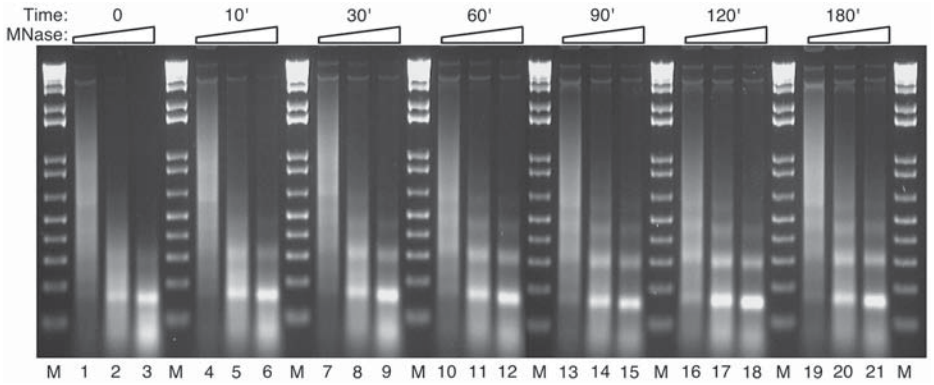

Fig. 2. Time-course of the chromatin assembly reaction assayed by micrococcal nuclease digestion. Assembly reactions were performed with CD fraction from wild-type cells and immediately digested with micrococcal nuclease (MNase, lanes 1–3), or allowed to incubate at 30°C for 10 (lanes 4–6), 30 (lanes 7–9), 60 (lanes 10–12), 90 (lanes 13–15), 120 (lanes 16–18), or 180 min (lanes 19–21) and then subjected to micrococcal nuclease digestion.

micrococcal nuclease digestion assays have been used to show that assembly is significantly impaired in CD fraction lacking histone chaperone Asf1 and the Snf2-like ATPase Chd1 ***(7)***. These results are consistent with the prevailing view that chromatin assembly can occur by a mechanism in which histone deposition is followed by a spacing step, which requires ATP ***(2)***. Replication-coupled assembly dependent on Asf1 may be regulated by cell-cycle cues and inhibited by DNA damage signals ***(1,2)***. Interestingly, the replication-independent assembly activity of the CD fraction does not differ between extracts from cells arrested at different stages of the cell cycle (**Fig. 3**) or between extracts of cells harvested before and after treatment with the DNA damaging agent methyl methanesulfonate (**Fig. 4**). These results raise the possibility that the replication-independent assembly pathway involving Asf1 is regulated differently than the replication-coupled assembly pathway dependent on Asf1.

3.7.3. Restriction Endonuclease Accessibility

1. To 15 µL of assembly, mix add 10 µL RNG buffer and 1 µL of restriction enzyme, incubate at 37ºC for 30 min (*see* **Note 11**).
2. Add 125 µL assembly stop solution + PK, incubate 20 min at 37ºC.
3. Extract with 150 µL phenol:chloroform, precipitate with 15 µL 2.5 *M* ammonium acetate and 340 µL ethanol.
4. Dry pellets, resuspend in appropriate buffer, and digest with second restriction enzyme and 0.1 mg/mL RNase A (*see* **Note 12**).
5. The entire sample is analyzed by agarose gel electrophoresis. An example of the expected result is shown in **Fig. 5**.

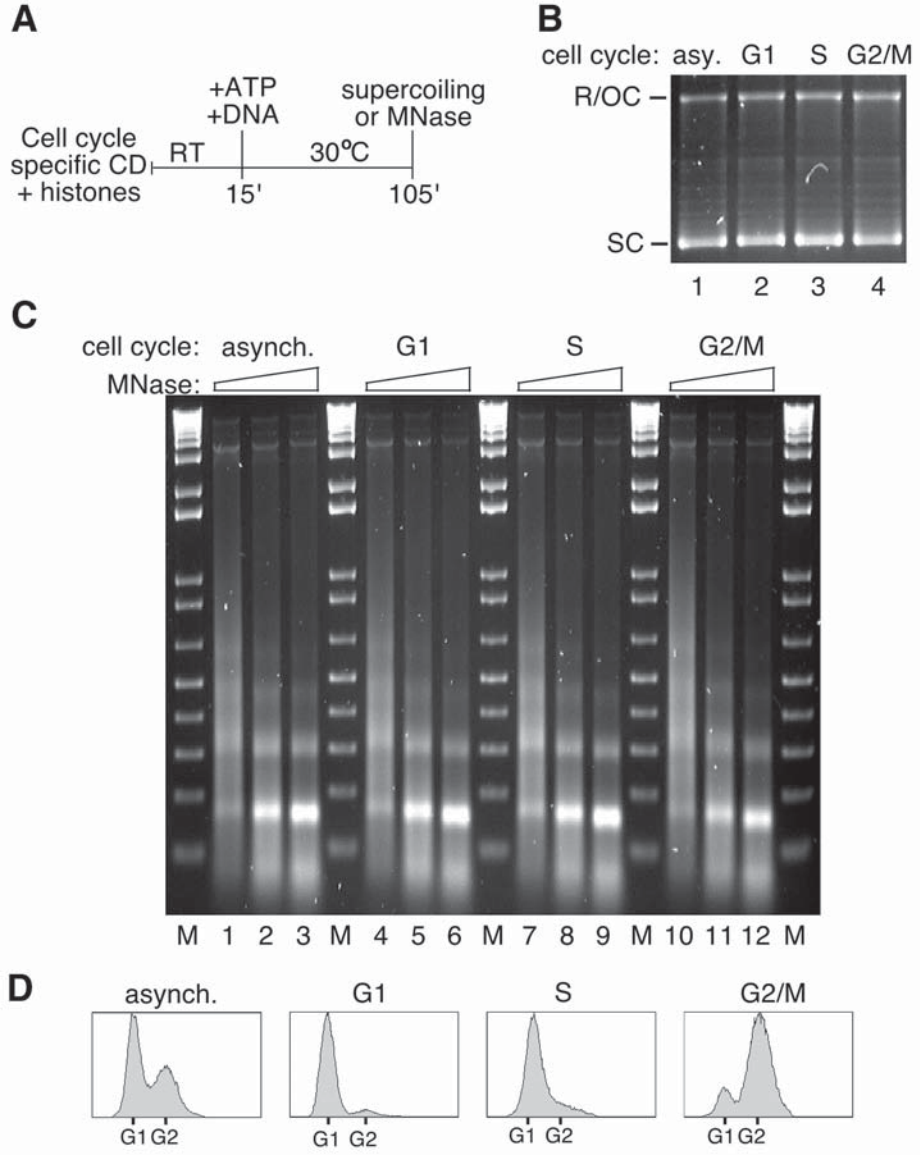

Fig. 3. Assays of chromatin assembly supported by CD fraction from wild-type yeast cells arrested at the indicated points in the cell cycle. After wild-type cells were grown overnight, they were resuspended in fresh pre-warmed media, and treated with nothing (asynchronously growing cells; Asyn.) or arrested with 100 ng/mL α-factor (G1), 0.2 *M* hydroxyurea (S), or 15 µg/mL nocadozole (G2/M). The outline of the experiment is shown **(A)**. Reactions contained 1 mg/mL extract protein, and the assembled products were analyzed by supercoiling assay **(B)** and by digestion with micrococcal nuclease (**C**; MNase). The DNA was resolved by agarose gel electrophoresis and visualized by staining with EtBr. Flow cytometry of the four different cultures is shown **(D)**. Flow cytometry was performed as described ***(15)***.

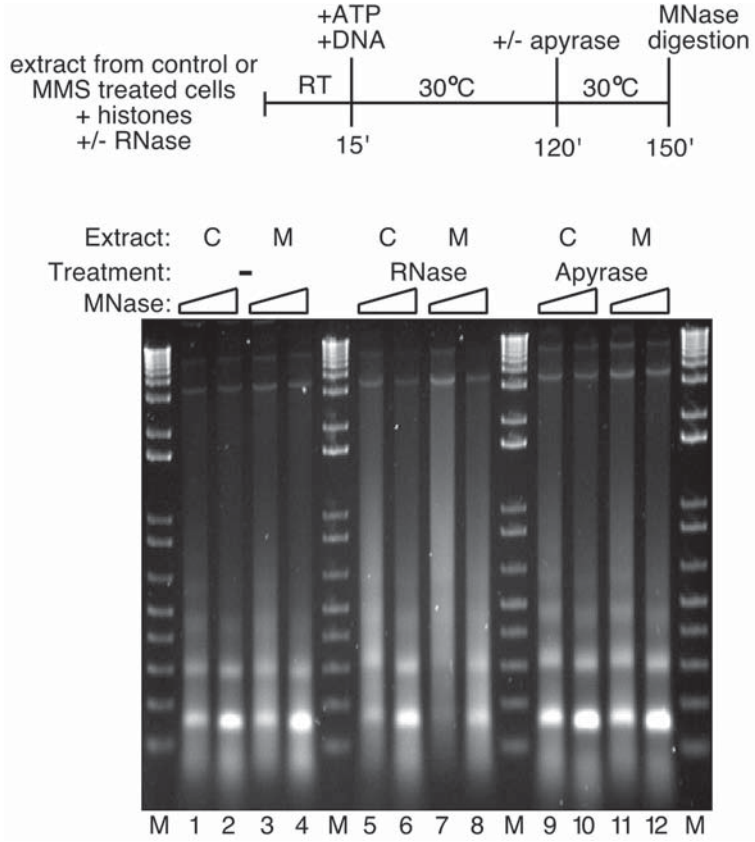

Fig. 4. Assays of chromatin assembly supported by CD fraction from wild-type yeast cells treated with the DNA damaging agent methyl methanesulfonate (MMS). Micrococcal digestions of chromatin samples made with assembly extracts prepared from untreated yeast cells (C) or from cells that had been treated with 0.075% MMS for 1 h immediately prior to spheroplasting (M). The extracts were used at a final protein concentration of 1.1 mg/mL in assembly reactions performed as normal (lanes 1–4), with pretreatment of the extracts with RNase A (0.12 µg/µL, lanes 5–8), or with addition of apyrase after 105 min of assembly (lanes 9–12).

4. Notes

1. Protease activity in yeast extracts can inhibit individual steps in the chromatin assembly reaction, especially because histone H3 is highly susceptible to proteolytic trimming. To minimize the recovery of proteases in the CD fraction, cells are grown in rich medium supplemented with ammonium sulphate, which represses the expression of some otherwise abundant proteases ***(12)***. The extraction and reaction buffers also include cocktails of standard protease inhibitors.

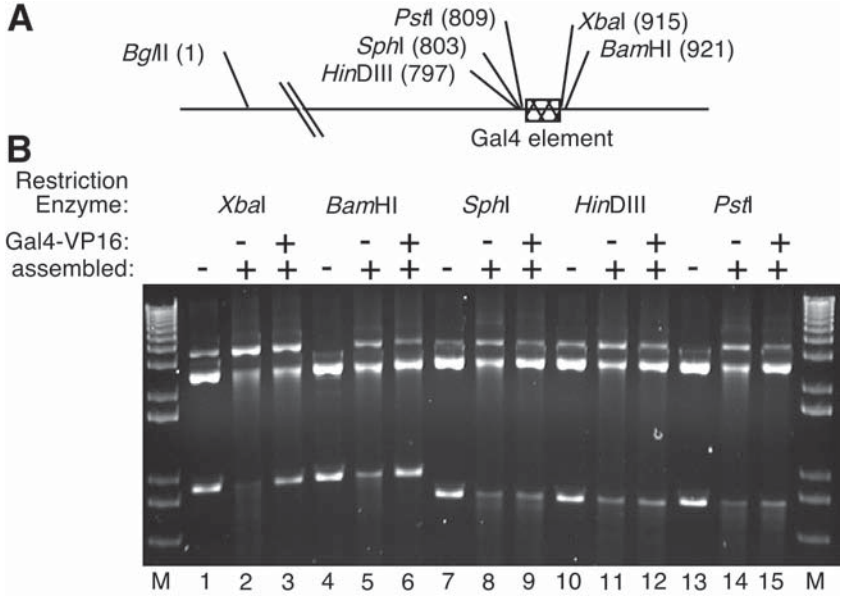

Fig. 5. Restriction endonuclease accessibility assay of chromatin assembly supported by CD fraction from a wild-type yeast strain. **(A)** Map of the 3.2 kbp plasmid with Gal4 elements (hatched box). Plasmid was assembled using the CD fraction with or without addition of Gal4-VP16 to a final concentration of 200 n*M*. **(B)** After assembly, the reactions and unassembled DNA (lanes 1, 4, 7, 10, and 13; these are controls for restriction enzyme activity) were digested with either *Xba*I (lanes 1–3), *Bam*HI (lanes 4–6), *Sph*I (lanes 7–10), *Hin*dIII (lanes 11–12), or *Pst*I (lanes 13–15). The DNA was deproteinized and digested with *Bgl*II.

When both these precautions are taken, active extracts are readily obtained from strains in which all protease genes are intact.

2. Frozen protease and phosphatase inhibitor stocks are stored in small aliquots.
3. Although stock creatine phosphate/ATP mix can be thawed repeatedly, creatine kinase should not be thawed more than twice.
4. This is a convenient point at which cells can be experimentally manipulated prior to extract preparation. For example, cell-cycle arrest can be imposed (as was done to obtain the results shown in **Fig. 3**) or cells can be treated with a DNA damaging agent (as was done to obtain the results shown in **Fig. 4**).
5. Two alternative methods for monitoring spheroplasting rely on direct observation by phase-contrast microscopy. First, the shape of the cell can be monitored; when they are spheroplasted, yeast cells have a rounder shape than usual (cells lose their normal egg-like shape). Secondly, the lysis of cells upon exposure to water can be monitored. In this case, cells are mounted under a coverslip and a

small amount of water is added to one edge of the coverslip. Partially spheroplasted cells will lyse to leave "ghost cells." Fully spheroplasted cells will lyse completely and therefore ghost cells will not be observed.

6. A polytron can also be used to break open the spheroplasts. A 1-min pulse of the polytron (Kinematica, distributed by Brinkmann Instruments, Westbury, NY) at setting 7 is given four times, with cooling on ice for 2 min between pulses of homogenization.
7. The lysis buffer is very viscous owing to the high concentration of Ficoll, making it difficult to recover all the homogenate from the homogenizer in the first draining. Therefore 2 to 3 mL of lysis buffer is reserved and used to collect the homogenate that remains in the homogenizer after the first draining.
8. The CD fraction can be prepared from spheroplasts or nuclei. In our hands, the CD fraction from spheroplasts has been perfectly adequate for chromatin assembly, and for the study of chromatin remodeling ***(7,13)***. However, it may be appropriate to test the CD fraction from nuclei when it is suspected that a cytoplasmic contaminant is interfering with assembly. For example, in a mutant in which an abundant cytoplasmic kinase is highly induced, aberrant phosphorylation of the chromatin assembly machinery might inhibit assembly.
9. A different temperature might be appropriate when assaying CD fraction from temperature-sensitive or cold-sensitive mutants.
10. Relaxed, closed circular template is prepared by incubation of plasmid DNA with DNA topoisomerase I or DNA topoisomerase II, under conditions specified for the enzyme being used. The amount of topoisomerase and length of time needed to relax template is determined empirically. Aliquots of the topoisomerase reaction can be directly added to assembly reactions.
11. RNG buffer is added so as to reduce the glycerol concentration (and thereby minimize star activity of the restriction enzyme), and to increase the Mg^{2+} concentration (which stimulates restriction enzyme activity). Even with the added magnesium, not all enzymes cut efficiently even in this buffer. Efficiency should be checked by cutting the same amount of unassembled plasmid under the same conditions. Typically, a restriction enzyme with one site in the plasmid is used; the rationale is outlined in **ref. *14*** and in **Note 12**.
12. A single-cutting enzyme is chosen so that the double digest of naked plasmid DNA yields two fragments that are easily resolved by agarose gel electrophoresis. If the site recognized by the first enzyme is protected when the plasmid is assembled into chromatin, then the yield of the smaller fragment (in particular) will be diminished compared to its yield in a double digest of naked DNA.

Acknowledgments

This work was supported by an operating grant from the Canadian Institutes of Health Research, and a Scientist award from the Alberta Heritage Foundation for Medical Research, to MCS.

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Chromatin Immunoprecipitation to Study Protein–DNA Interactions in Budding Yeast

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Summary

The accurate replication and expression of genetic information is ultimately governed by the interaction of regulatory proteins with specific sites on chromosomes. In recent years, our understanding of how these interactions occur in vivo has advanced considerably, in large part owing to the widespread application of chromatin immunoprecipitation (ChIP), a technique that allows quantification of protein–DNA interactions within the context of native chromatin. The ChIP assay involves three main steps: (1) chemical crosslinking of protein–DNA complexes in intact cells; (2) recovery of specific proteins by immunoprecipitation; and (3) detection of co-precipitating DNA sequences, usually by the polymerase chain reaction (PCR). Here, we provide a detailed description of a ChIP procedure that is commonly used to detect protein–DNA interactions in the yeast *Saccharomyces cerevisiae*, and discuss various methods for quantifying co-precipitating DNAs. This protocol and discussion should be particularly useful to those researchers interested in establishing ChIP assays in their laboratories.

Key Words: Chromatin; immunoprecipitation; ChIP; transcription factor; crosslinking; protein–DNA interaction; yeast.

1. Introduction

The chromatin (ChIP) assay provides a unique opportunity to determine whether a specific protein interacts with a particular piece of chromatin in vivo. Although the basic approach of using crosslinking agents to study protein–protein and protein–DNA interactions was reported as early as the 1960s ***(1,2)***, the present incarnation of the ChIP assay is a relatively recent advance, dating back to the early 1990s ***(3)***. Within the last 10 yr, there has been a tremendous increase in the number of researchers using this technique. This is especially true for those studying yeasts as a model organism, where powerful genetics,

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

combined with fully sequenced genomes, permit the analysis of virtually any protein–DNA interaction of interest. To date, an impression collection of proteins have been analyzed by ChIP, including RNA polymerases ***(4)***, histones ***(5,6)***, histone-modifying enzymes ***(5,7)***, transcription factors ***(8)***, silencing proteins ***(9)***, replication factors ***(10,11)***, and subunits of the proteasome ***(6,12)***. Collectively, these studies have played an instrumental role in unraveling mechanisms of transcription, chromatin modification, gene silencing, initiation of DNA replication, and cell-cycle control.

1.1. Chromatin Immunoprecipitation Overview

An overview of the ChIP technique is presented in **Fig. 1**. The first step in any ChIP protocol is the fixation of live cells with a nonspecific crosslinking agent, usually formaldehyde. Formaldehyde pentrates yeast cells rapidly, and aggressively crosslinks amino groups of nearby proteins and nucleic acids. The speed and efficiency with which formaldehyde works prevents the redistribution of chromosomal proteins during fixation, and allows efficient recovery of protein–DNA complexes during subsequent manipulations. Importantly, the ability of formaldehydye to produce both protein–protein and protein–DNA crosslinks means that proteins to be analyzed do not necessarily have to bind DNA directly, but rather can be crosslinked to DNA via other proteins such as the histones.

Following crosslinking, yeast are lysed, either by mechanical ***(13)*** or enzymatic ***(14)*** disruption, and soluble, crosslinked, chromatin prepared. A crucial step in the preparation of chromatin is sonication. Typically, crosslinked DNA fragments present in the initial cell lysate will be 20 kilobases (kb) or larger in length. The large size of these fragments is problematic, because proteins tend to interact with relatively small sites on DNA (<50 base pairs [bp]), and because 20 kb can span several genes in yeast. To allow precise mapping of the chromosomal location of any protein, it is thus necessary to cleave the chromatin into smaller fragments. Although a number of techniques have been used to fragment the chromatin, mechanical disruption by sonication is the preferred

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Fig. 1. *(opposite page)* Chromatin immunoprecipitation overview. Crosslinking: Yeast growing under appropriate conditions are treated with formaldehyde to induce protein–protein and protein–DNA crosslinks. Under these conditions, your protein of interest (YPI) is covalently cross-linked to chromatin in the vicinity of its target sequence, *A*, but not a distant reference sequence, *Z*. Sonication: Mechanical cleavage of chromatin by sonication results in the production of relatively small DNA fragments, one of which (*A*) is linked to YPI. Immunoprecipitation: YPI and cross-linked DNA sequence *A* are recovered by immunoprecipitation with antibodies specific to YPI. This process results in an enrichment of DNA fragments containing region *A*. DNA

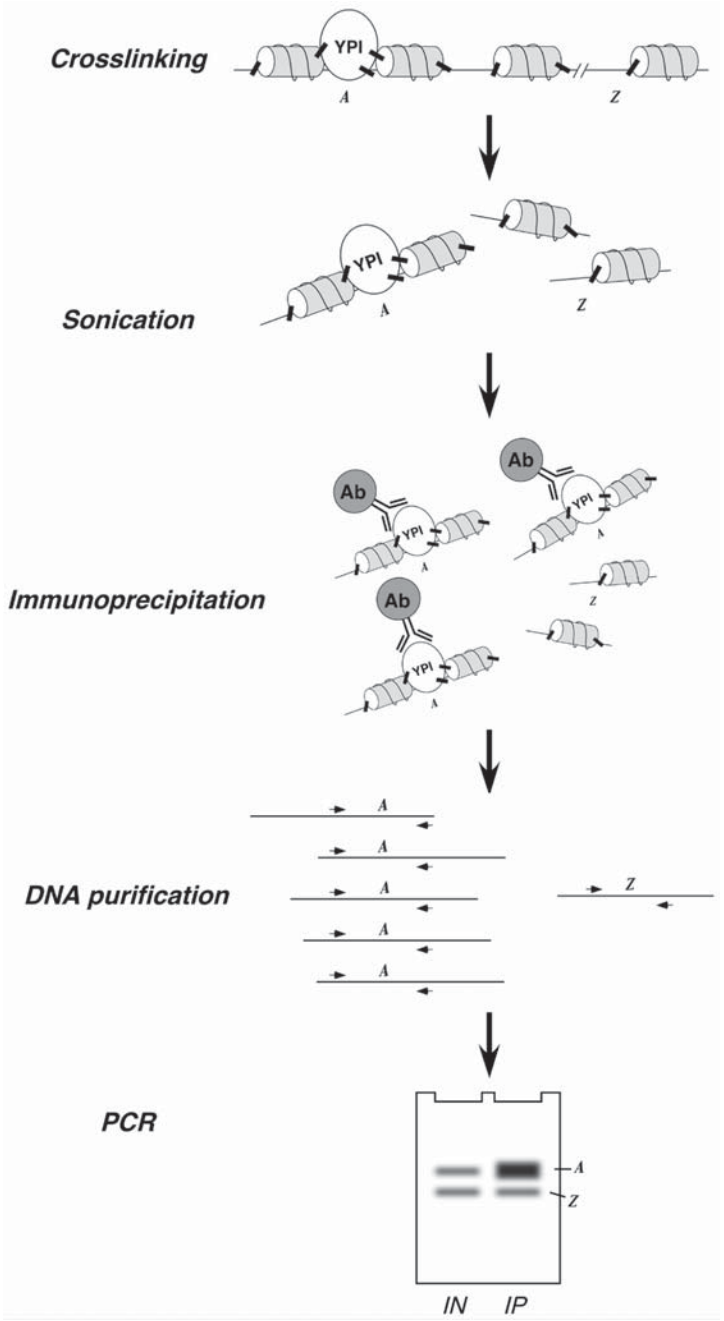

Fig. 1. *(continued)* purification: Following immunoprecipitation, DNAs are purified, and PCR used to quantify the levels of fragments corresponding region *A* vs region *Z*. The ratio of *A*/*Z* for the IP, relative to the same ratio from the input DNA (IN) gives a representation of the extent of binding of YPI to *A* in vivo.

method, because it can efficiently shear large DNA molecules into random fragments of approx 500 bp. Determining the efficiency of sonication is one of the most important parameters to establish before beginning a ChIP assay.

Finally, crosslinked protein–DNA complexes are recovered by immunoprecipitation with an antibody directed against the protein of interest. One of the particular advantages of studying yeast is that homologous recombination can be used to epitope tag a particular protein expressed from its own chromosomal locus. The development of rapid epitope-tagging techniques ***(15)*** has been important to the widespread use of ChIP because: (1) it allows analysis of proteins for which antibodies are not available; (2) it allows a diverse set of proteins to be assayed easily, by tagging with a set of standardized epitopes; and (3) it allows the specificity of the ChIP reaction to be quantified, by comparison of signal strength in immunoprecipitation reactions performed in tagged vs untagged yeast strains.

1.2. Analysis of Co-Precipitating DNAs

After recovery of specific protein–DNA complexes, crosslinks are reversed by heat treatment, and co-precipitating DNAs recovered by phenol-chloroform extraction and ethanol precipitation. A variety of techniques have been used to detect specific DNA fragments in the precipitated material, including Southern hybridization and dot blotting. Typically, however, the amount of specific DNA recovered in a ChIP is small, and polymerase chain reaction (PCR)-based techniques—in which precipitated DNAs are used as template for amplification with specific sets of primers—are most commonly used to analyze ChIP DNA.

Here, we detail two PCR-based methods for quantifying precipitated DNAs: standard PCR and real-time, quantitative, PCR (Q-PCR). In both methods, it is essential that amplification reactions are quantitative; that is, that the amount of amplified material is directly proportional to the amount of input DNA. It is also important that reference DNA sequences, not expected to bind the protein of interest, are amplified as part of the analysis, to assess the level of nonspecific ("background") binding to chromatin. Use of standard PCR has the advantage of employing common lab equipment and reagents, such as a basic thermocycler and thermostable polymerase. With this approach, however, an extensive series of controls are needed to ensure that the PCR amplification is quantitative, and radioactivity is often required for precise quantification of amplified products. Frequently, ChIP DNAs must be analyzed under several different amplification conditions to insure a quantitative PCR reaction. Real-time PCR, in contrast, requires a significant investment in equipment—i.e., purchase of a thermocycler capable of detecting incorporation of a fluorescent dye *during* the amplification reaction—but offers the advantage of being able to precisely quantify levels of DNA during the exponential phase of the

amplification reaction. For many, the added expense of Q-PCR is justified by the ability to consistently and accurately measure co-precipitating DNAs in a single PCR reaction.

2. Materials

2.1. Solutions

1. DOC Buffer: 10 m*M* Tris-HCl, pH 8.0, 0.25 *M* lithium chloride, 0.5% Nonidet-P40 (NP-40), 0.5% sodium deoxycholate (DOC), 1 m*M* ethylenediaminetetraacetic acid (EDTA, pH 8.0). Sterilize through 0.45-µm filter. Store at room temperature.
2. 2.5 *M* Glycine. Adjust to pH 8.0 with 10 *N* sodium hydroxide to dissolve. Sterilize through 0.45-µm filter. Store at room temperature.
3. Lysis Buffer: 50 m*M* HEPES, pH 7.5, 500 mM sodium chloride, 1 m*M* EDTA, pH 8.0, 1% Triton X-100, 0.1% DOC, 0.1% sodium dodecyl sulfate (SDS). Sterilize through 0.45-µm filter. Store at 4°C. Immediately before use, add proteinase inhibitors: 0.4 mg/mL Pefablock (Roche), 10 µg/mL Leupeptin, 10 µg/mL Pepstatin, 5 µg/mL Aprotinin.
4. Phosphate-buffered saline (PBS): 137 m*M* sodium chloride, 2.7 m*M* potassium chloride, 10 m*M* disodium hydrogen phosphate, 2 m*M* potassium dihydrogen phosphate. Adjust to pH 7.4 with 10 *M* hydrochloric acid. Sterilize through 0.45-µm filter. Store at 4°C.
5. Proteinase K: 10 mg/mL proteinase K, dissolved in sterile water. Store in aliquots at –20°C.
6. 3.0 *M* Sodium acetate. pH 4.8. Autoclave. Store at room temperature.
7. TE: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0. Autoclave. Store at room temperature.
8. TES: 50 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, pH 8.0, 1% SDS. Sterilize through 0.45-µm filter. Store at room temperature.

2.2. Reagents

1. Complete Proteinase Inhibitor Cocktail, 20 tablets (Roche, Indianapolis, IN; cat. no. 1 697 498).
2. 37% Formaldehyde Solution (Fisher Scientific, Pittsburgh, PA; cat. no. BP531-500).
3. Protein A agarose (Roche; cat. no. 1 134 515).
4. Protein G agarose (Roche; cat. no. 1 243 233).
5. SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA; cat. no. 4309155).
6. Anti-HA, mouse (12CA5) (Roche; cat. no. 1583816).
7. Anti-Myc (9E10), mouse (Upstate Biotechnology, Charlottesville, VA; cat. no. 05-419).
8. IgG agarose, rabbit (Sigma, St. Louis, MO; cat. no. A2909).
9. Anti-Flag (M2), mouse (Sigma; cat. no. F3165).

2.3. Supplies

1. Acid-washed glass beads, 425-600 µ (Sigma; cat. no. G-8772). Store in refrigerator.
2. Mini Bead Beater (BioSpec Products, Bartlesville, OK; cat. no. 693).
3. 96-well optical plate (Applied Biosystems; cat. no. N801-0560).
4. Optical caps (Applied Biosystems; cat. no. 4323032).
5. Safe-lock tubes, 1.5 mL (Eppendorf, Westbury, NY; cat. no. 22 36 320-4).
6. Siliconized, flat-cap tubes, 1.5 mL (Fisherbrand; cat. no. 02-681-320, Fisher Scientific).
7. Siliconized 2-mL screw-cap tubes, O-ring seal (Fisherbrand; cat. no. 05 669 9).
8. 0.5-mL Theromo-tube for PCR (distributed by Marsh, Rochester, NY; cat. no. AB-0489).
9. Ultrasonic processor (Sonics and Materials, Inc., Newtown, CT; cat. no. VC 130 PB) equipped with stepped microtip (Sonics; cat. no. 630-0422).
10. DNA Engine Opticon Continuous Fluorescent Detection System (MJ Research, Waltham, MA; cat. no. CFD-3200).

3. Methods

Many excellent ChIP protocols have been published, both in the literature and on the internet. Despite the wealth of protocols available, however, establishing a successful ChIP assay can be a daunting task. We have therefore written this chapter specifically for those interested in performing ChIP for the first time. ChIP is a relatively protracted procedure; there are several stages at which problems can be encountered, and there are a few operations that need to be optimized to ensure a successful outcome. Here we begin with a discussion of parameters that need to be optimized *before* proceeding with the ChIP assay; these important parameters include sonication conditions, choice of antibodies and epitope tags, and selection of appropriate controls. We then present a ChIP protocol that is routinely used in our laboratory to study interaction of transcriptional regulators with chromatin (e.g., **ref. *8***). This protocol should be a good starting point for most ChIP assays. Finally, we describe two alternate procedures for analyzing co-precipitated DNAs: by standard PCR or Q-PCR. Together, these descriptions should provide first-time ChIP users with enough information to get started.

3.1. How to Start

Owing to the complexity and protracted nature of the ChIP protocol, it is desirable that certain parameters, crucial to the success of the procedure, be optimized prior to using a ChIP assay in any experiment. Here we discuss the four most critical parameters: immunoprecipitation, sonication, control design, and PCR analysis.

3.1.1. Immunoprecipitation

The success of any ChIP assay ultimately depends on the specificity and reactivity of antibodies used to capture the protein of interest. Two general types of approaches can be used: (1) tagging of endogenous genes with epitope tags, and (2) use of antibodies against native proteins. The advent of rapid gene-tagging techniques for yeast ***(15)*** has greatly accelerated the use of ChIP analyses, because, theoretically, any protein can be studied without the time or expense required to raise antibodies against native proteins. Moreover, a standard set of tags and matching antibodies can be developed, such that, once optimized, numerous proteins can be assayed quickly by tagging with a reliable and robust epitope tag. Typical tags include 3×HA, which is recognized by antibody 12CA5 (Roche; cat. no. 1583816); 3×Myc, which is recognized by antibody 9E10 (Upstate Biotechnology; cat. no. 05-419); TAP, which binds directly to IgG agarose beads (Sigma; cat. no. A2909); and Flag, which is recognized by antibody M2 (Sigma; cat. no. F3165). We, however, do not use the Flag epitope tag in our experiments, because of considerable cross-reactivity with some (as yet unknown) chromatin-associated protein (unpublished observations). It is also important to determine the amount of antibody to use for immunoprecipitation. We recommend trying different amounts of antibody in a pilot ChIP experiment. The major downside of using epitope tags is that tagging a protein, even one expressed from its endogenous locus, can alter its function. The histone methyltransferase Set1, for example, is inactivated by carboxyl-terminal tagging ***(16)***, which is the most common position for tagging genes in yeast. Thus, when employing tagging methods, it is important to make sure that the presence of the epitope tag does not substantially interfere with the functional characteristics of the protein.

Antibodies raised against specific protein antigens can offer selectivity and high signal strength, but usually require affinity purification prior to use (most animal hosts have endogenous antibodies against yeast proteins that will yield high background signals). The best strategy, if possible, is to use polyclonal antibodies (PAbs) that have been raised against recombinant proteins. Although monoclonal and anti-peptide polyclonal antibodies can work well, relying on a single epitope to precipitate a protein in this kind of assay can be troublesome, because accessibility of the epitope within a crosslinked chromatin complex can be limited. As a general rule, PAbs (or mixtures of monoclonals) offer the best probability of finding an epitope on the target protein.

3.1.2. Sonication

One of the great advantages of ChIP is the ability to relate a chromatin-associated protein to a specific site on the DNA in vivo. Because PCR-based detection methods only ask if a specific piece of DNA is present in the IP—

they do not give information about the size of the fragment that contained that piece of DNA—the resolution of ChIP is ultimately determined by the size of the DNA fragments being co-precipitated. If the fragments are too large, then the signal generated by amplification of a specific DNA segment could actually come from binding of the protein to a site several kb away! It is thus essential that sonication—the process used to mechanically shear the DNA—be optimized to generate fragments of the appropriate size: 0.5–1.0 kb for standard PCR, 0.1–0.5 kb for Q-PCR.

Exact sonication conditions will depend on the instrument and the tip being used. We recommend that a series of different sonication conditions be tested, beginning by simply altering the number of rounds of sonication the samples receive. Note that it is important that sonication conditions be optimized for crosslinked samples under ChIP conditions, and that crosslinks be reversed prior to analysis of DNA size. Optimization steps are:

1. Follow steps in **Subheadings 3.2.–3.4.** of the ChIP protocol for one 50-mL culture.
2. Prior to sonication, transfer 50 µL of lysate to a microfuge tube labeled "0," and place aside. This is the "unsonicated" control.
3. Subject the remaining sample to a single 10-s pulse of sonication as described in **Subheading 3.5.** Transfer 50 µL of lysate to a microfuge tube labeled "1," and place aside.
4. Subject the remaining sample to a single 10-s pulse of sonication as described. Transfer 50 µL of lysate to a microfuge tube labeled "2," and place aside.
5. Continue this process until <50 µL of lysate remains.
6. Add 150 µL of TES to each sample. Reverse crosslinks, purify, and precipitate DNA as described in **Subheading 3.8., steps 4–13**.
7. Analyze 50 µL of each DNA sample by agarose gel electrophoresis and ethidium bromide (EtBr) staining. The DNA will run as a smear, the average size of which will decrease with increased sonication conditions.

3.1.3. Control Design

Before beginning any ChIP experiment, one should think carefully about appropriate controls. When starting ChIP for the first time, a positive control is a useful way to determine whether the ChIP procedure is working. We recommend using a commercially available antibody against RNA polymerase II (8WG16: Covance Research Products) that consistently performs well in ChIP, and should yield a strong signal with any transcriptionally active mRNA-encoding gene. Given the real problems that can be encountered with antibody cross-reactivity (both specific and nonspecific), it is also important to include an appropriate series of negative controls. If antibodies were generated "in-house," it is usually possible to obtain pre-immune serum. If this option is not available, negative controls can include using either no antibody in the immu-

noprecipitation, or using an antibody from an unrelated protein that is not expected to bind to chromatin. Of course, if epitope-tags are being used, the best, and essential, negative control is to perform ChIP assays, under identical conditions, with a congenic strain that lacks the epitope tag. In this way, issues of antibody cross-reactivity are normalized between samples, and ChIP signal can be directly attributed to the epitope-tagged protein. Finally, it is crucial to include appropriate controls for analysis of co-precipitated DNAs. Even if antibodies are highly specific, inappropriate DNA molecules may be brought down in the ChIP assay owing to nonspecific binding to antibodies, beads, or the plastic of the tubes. To control for nonspecific DNA recovery, we include in our PCR analyses a set of primers designed to amplify a control (or reference) DNA fragment at which the protein of interest is not expected to bind. In analysis of transcriptional activation, for example, researchers often use a nontranscribed (or even silenced) region of the genome as a reference point to which all experimental ChIP signals are normalized.

3.1.4. PCR Analysis

For analysis of ChIP DNA by standard PCR, it is important to select primers that amplify their target sequences efficiently, and to test amplification on genomic DNA (usually INPUT DNA) prior to performing an experiment. If the specific binding site of a protein on DNA is known, the target sequence for amplification can be centered on this site. If the binding site is not known, then it is useful to amplify a series of target sequences, spaced 500–1000 bp apart, to empirically determine the DNA fragment that gives the most robust ChIP signal. Optimal fragment size for amplification is around 200 bp.

A common approach, and one that economizes use of precious ChIP DNA, is to perform multiplex PCR, in which gene-specific and reference primers are amplified in the same tube. When performing multiplex PCR, the gene-specific and reference primers should have similar melting temperatures, and should amplify fragments of a slightly different size (<40 bp difference), so that the two fragments can be distinguished by gel electrophoresis. Pilot experiments should be performed, using INPUT DNA samples, to determine PCR conditions that will amplify both the gene-specific and reference DNA fragments with approx equal efficiency.

Finally, note that it is absolutely essential that PCR conditions be quantitative; in other words, that a twofold increase in the level of a target sequence in the PCR reaction will result in a twofold increase in PCR product yield. In practice, it can be difficult to balance signal intensity (which increases with PCR cycle number) vs linearity (which will decrease with PCR cycle number). For each primer set, we recommend performing a fairly detailed preliminary

analysis on INPUT DNA, varying PCR cycle number, to establish a cycle number that will give good signal strength, while at the same time accurately reflecting the relative amount of target sequence.

3.1.5. Quantitative PCR

Many of the considerations that apply to standard PCR—choice of reference vs gene-specific target "amplicons," direct determination of the quantitative nature of the reaction—also apply to Q-PCR. The main concern with Q-PCR is the nature of the primers themselves. Primers must be selected to minimize nonspecific products or primer-dimers. For our Q-PCR analyses, we amplify small fragments; 70–150 bp in length. The primers should not end in a G residue, three out of the last five nucleotides should be an A or a T, and the primers should not contain runs of identical nucleotide residues greater than 5 bp in length. Even if these recommendations are followed, it is still important to determine the specificity of amplification prior to performing an actual experiment. This can be done by performing "no DNA" PCR control reactions, and by directly analyzing the products of Q-PCR reaction by agarose gel electrophoresis, to determine that a signal band of the correct size is amplified.

Below we provide a standard ChIP protocol that is routinely used in our laboratory.

3.2. Cell Growth

1. Inoculate fresh 5-mL starter cultures of each yeast in appropriate media. Grow overnight at the appropriate temperature (usually 30°C) (*see* **Note 1**).
2. Next day, inoculate 50 mL of appropriate selective media (in 250-mL flask) with approx 1/200 dilution of the original starter culture. Grow at the appropriate temperature overnight until the optical density (OD) of the culture (at 600 nm) is approx 0.8–1.0. (*see* **Note 2**).

3.3. Crosslinking

Prepare: Ice-cold PBS; benchtop centrifuge at 4°C; and labeled 50-mL Falcon tubes, one for each culture.

1. Transfer flasks to rotating platform at room temperature. Rotate slowly.
2. With culture slowly rotating, add 1.4 mL of 37% formaldehyde dropwise to each culture (final concentration 1%). Continue to rotate for 15 min (*see* **Note 3**).
3. Add 3 mL of 2.5 *M* glycine to each culture. Continue to rotate for 5 min at room temperature.
4. Transfer cell suspension to a labeled 50-mL conical tube. Spin at 800*g* for 5 min at 4°C. Pour off supernatant. Wash cell pellet twice in 50 mL ice-cold PBS. Proceed immediately with lysis or snap-freeze pellets in liquid nitrogen and store at –70°C.

3.4. Cell Lysis

It is important to keep samples cold throughout the procedure (perform on ice or in cold room). Prepare: Labeled 2-mL siliconized screw-cap tubes, one for each culture; ice-cold lysis buffer + freshly added inhibitors (400 µL per sample); labeled 1.5-mL siliconized microfuge tubes (remove caps), one for each culture; and labeled 1.5-mL siliconized microfuge tubes (caps), one for each culture.

1. Resuspend cell pellet in 400 µL of ice-cold Lysis Buffer and transfer to a siliconized 2-mL screw-cap microfuge tube (*see* **Note 4**).
2. Add 500 µL cold, acid-washed, glass beads to each sample (*see* **Note 5**).
3. Place tubes into BioSpec bead beater set up in the cold room. Disrupt cells with 4 ×40-s pulses, with the bead beater set to "homogenize." To avoid heating the samples, allow a 1-min rest on ice between each pulse (*see* **Note 6**).
4. Puncture the bottom of the 2-mL screw-cap tube with an 18-G needle. Place this tube into a 1.5-mL siliconized microfuge tube (with no cap), and place both tubes inside a 15-mL conical centrifuge tube (*see* **Note 7**).
5. Spin the tubes in the benchtop centrifuge at 800*g* for 5 min at 4°C.
6. Collect the soluble lysate that has spun through into the lower microfuge tube and transfer it to a fresh 1.5-mL siliconized microfuge tube. Place samples on ice.

3.5. Sonication

Prepare: Labeled 1.5-mL siliconized microfuge tubes, one for each sample.

1. Sonicate each lysate (setting "5") for 10 s. Keep samples on ice as much as possible. Repeat sonication four more times for a total of 50 s. Note that samples are disrupted in a series of 10-s pulses (as opposed to a continuous 50-s pulse) to avoid heating (*see* **Note 8**).
2. Centrifuge all tubes at 16,000*g* in microcentrifuge for 5 min at 4°C. Transfer supernatant to a fresh 1.5-mL siliconized tube. Proceed immediately with immunoprecipitation or snap-freeze lysates in liquid nitrogen and store at –70°C.

3.6. Immunoprecipitation

Prepare: Two sets of labeled 1.5-mL siliconized microfuge tubes; one set labeled "INPUT." Ice-cold lysis buffer + freshly-added inhibitors (50 µL per sample). Protein A agarose/Protein G agarose (40 µL per sample), prepared fresh before using as follows: Mix 20 µL of Protein A agarose with an equal volume of Protein G agarose. Wash with 50 volumes of Lysis Buffer and resuspend in 1 volume of Lysis Buffer (+ inhibitors).

1. To each sample, add 40 µL of Protein A/G agarose. Place tubes on a rotator for 1 h at 4°C. (*see* **Note 9**).
2. Spin tubes at 400*g* in a microcentrifuge for 3 min at 4°C. Transfer supernatant into a fresh siliconized microfuge tube.

3. Remove 50 µL (1/10 of total volume) from each sample and transfer to fresh 1.5-mL siliconized microfuge tubes labeled "INPUT." Keep INPUT tubes at –20°C until steps in **Subheading 3.8.**
4. To remaining lysates, add appropriate antibody for immunoprecipitation (*see* **Note 10**). Incubate reactions overnight at 4°C on rotator.
5. Next day, spin tubes briefly and transfer supernatant to a new 1.5-mL siliconized microfuge tube.
6. Add 40 µL of freshly prepared protein A/G agarose mix. Rotate tubes for 1 h at 4°C to collect immune complexes (*see* **Note 11**).

3.7. Washing the Immunoprecipitates

Prepare: Lysis Buffer + Complete Inhibitors. Use 3 mL per IP. Dissolve 1 complete protease inhibitors tablet per 25 mL. Labeled 1.5-mL siliconized microfuge tubes, one for each IP.

1. Collect immune complexes by spinning tubes at 400*g* for 3 min in a microcentrifuge at 4°C.
2. Carefully remove the supernatant by aspiration, trying not to disturb the beads. To avoid crosscontamination, fit the aspirator with a disposable tip (e.g., micropipet tip), and change after each sample (*see* **Note 12**).
3. Wash immune complexes by adding 1 mL of Lysis buffer, rotating sample for 5 min at room temperature, and collecting beads as described immediately above.
4. Repeat the wash in Lysis Buffer.
5. Wash once in 1 mL DOC buffer at room temperature.
6. Wash once in 1 mL of TE at room temperature.
7. After the final wash, resuspend beads in 0.5 mL of TE, and transfer to a fresh 1.5-mL siliconized microfuge tube. Additionally, add 0.5 mL of TE to the previous tube to capture any remaining beads, and combine with the 0.5 mL in the fresh tube.
8. Collect beads by centrifugation at 400*g* for 3 min in a microcentrifuge at room temperature. Carefully remove supernatant with pipet. Remove any final traces of liquid by fitting the tip of an aspirator with a 26-G needle, and plunging the needle directly into the beads. Proceed immediately to **Subheading 3.8.**

3.8. Reverse Crosslinking

Prepare: Labeled siliconized tubes, one per IP; labeled safe-lock microfuge tubes, three tubes per IP. Set up 65°C water bath.

1. Add 50 µL of TES to the beads, mix briefly by vortexing, and incubate the tubes at 65°C for 10 min.
2. Centrifuge tubes in a micro centrifuge for 5 min at 16,000*g* at room temperature. Transfer supernatant to a fresh safe-lock tube and save.
3. Add an additional 150 µL of TES to the beads. Vortex, and pellet beads by centrifugation as described in **step 2**. Transfer supernatant to the tube in **step 2**. Incubate tubes at 65°C overnight (*see* **Note 13**).

4. Add 150 µL of TES to the INPUT tubes from **Subheading 3.6., step 3**. Incubate tubes at 65°C overnight.
5. Next day, allow tubes to cool. Spin briefly. Transfer liquid to a fresh 1.5-mL safe-lock microfuge tube containing 25 µL of 10 mg/mL proteinase K and 200 µL of TE. Incubate at 37°C for 2 h.
6. Add 400 µL of 25:24:1 phenol/chloroform/isoamyl alcohol to each sample. Vortex for 30 s. Centrifuge in microcentrifuge at 16,000*g* for 10 min at room temperature.
7. Transfer aqueous (upper) layer to fresh 1.5-mL safe-lock tube. Add 400 µL of chloroform to each sample. Vortex for 30 s. Centrifuge in micro centrifuge at 16,000*g* for 10 min at room temperature.
8. Transfer aqueous (upper) layer to fresh 1.5-mL safe-lock tube containing 44 µL of 3 *M* sodium acetate and 20 µg of glycogen (*see* **Note 14**).
9. Precipitate DNA by the addition of 1 mL of ice-cold 200-proof ethanol. Mix and place at –20°C overnight.
10. Collect DNAs by centrifugation in a microfuge at 16,000*g* for 30 min at 4°C. Carefully remove supernatant.
11. Wash DNA pellet with 500 µL of ice-cold 70% ethanol, and carefully remove as much liquid from the pellet as possible. Allow DNA pellet to dry by leaving tube open to the atmosphere for approx 30 min.
12. Resuspend IP DNA in 100 µL of TE.
13. Resuspend INPUT DNA in 500 µL of TE.
14. Store all DNA samples at –20°C until analysis by either standard PCR or Q-PCR. Both methods are described in **Subheadings 3.9.** and **3.10.**

3.9. Standard PCR Analysis

1. Prepare a dilution set of one of the INPUT DNA samples to determine whether PCR amplification is quantitative. Dilute INPUT DNAs 1:50, 1:100, 1:200, 1:400, and 1:800 in water. Use filtered micropipet tips for all manipulations.
2. Prepare enough PCR Master Mix for all reactions (*see* **Note 15**).

Solution	Final concentration
10X Taq buffer (no $MgCl_2$)	1X
$MgCl_2$ (25 m*M*)	2 m*M*
dNTPs (25 m*M*)	200 µ*M*
α(P^{33}-dCTP)	2.5 µCi
Primer #1 (20 µ*M*)	0.5 µ*M*
Primer #2 (20 µ*M*)	0.5 µ*M*
Taq polymerase	2.5 U
Water	To 23 µL

3. Aliquot 23 µL of Master Mix solution into 0.5-mL PCR tubes. Store tubes on ice.
4. Transfer 2 µL of each IP DNA, and 2 µL of each dilution of INPUT DNA, to the tubes containing the PCR master mix.

5. Transfer tubes to thermocycler. Cycle parameters are: initial denaturation for 5 min at 95°C, followed by 20–25 cycles with 1 min at 95°C (denaturation), 1 min at 50°C (annealing), 1 minute at 72°C (elongation), and a final extension step of 10 min at 72°C (*see* **Note 16**).
6. Following amplification, the labeled products are resolved by polyacrylamide gel electrophoresis (PAGE) and subsequently visualized and quantified by phosphorimaging (*see* **Note 17**).

3.10. Quantitative Real-Time PCR

1. All DNAs are assayed in triplicate. Prepare enough Q-PCR Master Mix for all reactions (*see* **Note 18**).

Solution	Amount
SYBR Green PCR Master Mix	6.25 µL
Primer 1-1 (5 µ*M*)	0.75 µL
Primer 1-2 (5 µ*M*)	0.75 µL
Water	3.75 µL (Total 11.5 µL per sample)

2. Aliquot Master Mix Q-PCR into wells of 96-well Optical plate.
3. Add 2 µL of appropriate DNA template into each well. Be sure to include "no template" control reactions that receive 2 µL TE instead of template DNA.
4. Cover 96-well plate with strips of Optical caps. Vortex entire plate to mix, and spin briefly in benchtop centrifuge. Proceed immediately with PCR.
5. Place samples in real-time thermocycler. Cycle parameters are: 95°C for 10 min (denaturation), followed by 40 cycles of 94°C for 15 s (denaturation) and 60°C for 1 min (annealing).
6. After completing PCR cycles, for each reaction, determine the cycle threshold (*C*) (*see* **Note 19**). Average the cycle threshold for each triplicate set of reactions. For calculation, name average cycle number for IP DNA/Primer Set *X* as C_X^{IP}; IP DNA/Reference Primer Set as C_{Ref}^{IP}; INPUT DNA/Primer Set *X* as C_X^{IN}; and INPUT DNA/Reference Primer Set as C_{Ref}^{IN}.
7. Calculate fold enrichment (**F**) for each IP sample using following formula:

$$F = [2^{-(C_X^{IP} - C_{\mathrm{Ref}}^{IP})}]/[2^{-(C_X^{IN} - C_{\mathrm{Ref}}^{IN})}]$$

4. Notes

1. Yeast strains carrying mutations, or expressing epitope-tagged proteins, can grow more slowly than congenic wild-type strains. If so, adjust growth time to compensate.
2. It is important to perform crosslinking on yeast cultures at approx the same OD (OD_{600} = 0.8–1.0). Sometimes it is helpful to set up several overnight inoculates with different dilutions of the starter culture (e.g., 1/100, 1/200, 1/400) in order to achieve desired OD for all cultures at the same time. If an induction step is required (such as galactose or copper induction), treat cells so that, when the

induction is complete, the yeast are at the correct density. Note that many DNA–protein interactions are transient, and that the exact point after induction at which crosslinking is performed may be important. If this is a concern, perform a time-course experiment to empirically determine the optimal time for crosslinking. Because yeast are dead almost immediately following exposure to formaldehyde, it is possible to perform a fairly detailed time-course using this technique.

3. Keep a separate bottle of formaldehyde for ChIP experiments. Note that formaldehyde is toxic; use appropriate handling techniques. The time and temperature of fixation can be altered to optimize signal strength. Less robust interactions may be revealed by performing the crosslinking at lower temperatures, or by increasing the amount of time yeast are exposed to formaldehyde. We recommend starting with 15 min at room temperature. If this does not work, increase crosslinking time to 60 min. If this fails, try 16°C for the fixation temperature. Note that sonication conditions are specifically linked to crosslinking conditions, and will need to be re-optimized if crosslinking conditions change.
4. The use of siliconized tubes is essential to minimize nonspecific binding of DNA and proteins to the plastic, which in turn will give high background signals. Microfuge tubes can be siliconized in the laboratory, but for consistency and convenience we recommend purchasing them pre-treated.
5. To measure glass beads accurately, prepare a scoop by cutting a microfuge tube at the 500 µL mark and attaching it to an 18-G needle as a handle.
6. An alternate method for preparing cell lysates involves the production of spheroplasts ***(14)***. This technique involves more time than the one presented here, but it does work well.
7. This procedure is designed to separate the cell lysate quickly from the glass beads and from the insoluble yeast material. Other methods can be used, but the particular advantage of this technique is that it completely separates the lysate from the beads without the need to wash the beads (which would increase the volume of the lysate).
8. As mentioned in **Subheading 3.1.**, sonication conditions have to be optimized in advance, and are specific for each instrument and configuration. The numbers presented here are a good starting point, but they are specific for the sonicator we use in the laboratory (Sonics, VC 130 PB).
9. This is a pre-clearing step designed to reduce nonspecific capture of proteins by the Protein A/G resin. Although it is not included in most ChIP protocols, we find it valuable in reducing background signal in ChIP reactions. Note that we use a mixture of Protein A and G resins because, individually, each has subtle specificities for different antibody types; the combination of the two leads to robust antibody capture, regardless of antibody subtype.
10. It is important to optimize the exact amount of antibody used in each IP reaction to insure that the antigen is being quantitatively recovered. We suggest performing IP reactions (under ChIP conditions) with increasing amounts of antibody, and using Western blotting to determine the amount of antibody that results in near-complete depletion of antigen from the lysate (measured at the end of **Sub-**

heading 3.6.). It is also important to optimize the time of immunoprecipitation. Overnight is convenient, but it can raise background signals. We recommend trying different times of incubation in initial experiments to determine the best conditions.

11. If TAP-tagged proteins are to be analyzed by ChIP, **Subheading 3.6.** in the ChIP protocol will need to be modified, because TAP-tagged proteins carry the Protein A tag, and must be recovered on rabbit IgG agarose (A-2909, Sigma). For this reason, binding of antibody to antigen and collecting antibody/antigen complexes steps can be combined. The alternate procedure that replaces **Subheading 3.6.** of the protocol is:
    TAP-1: Remove 50 µL (1/10 of total volume) from each sample and transfer to fresh 1.5-mL siliconized microfuge tubes labeled "INPUT." Keep INPUT tubes at –20°C until **Subheading 3.8.**
    TAP-2: To each sample, add 40 µL of rabbit IgG agarose, prepared by washing three times in 400 µL of Lysis Buffer.
    TAP-3: Rotate tubes for 2 h at 4°C to collect immune complexes.
    TAP-4: Continue with standard protocol at **Subheading 3.7.**
12. ChIP is a technique that involves the selective elimination of most DNA sequences from a complex mixture. Cross-contamination of samples is therefore a real concern with this assay, and can ruin many days worth of experimentation. Do not take any chances. Use fresh, plugged, microfuge tips for all additions; change tips for washing. Clean pipets and equipment frequently.
13. We recommend sealing lids of microfuge tubes with laboratory film before placing them into the 65°C incubator to prevent loss of sample or contamination from tubes inadvertently opening.
14. It is important to completely remove all proteins at this stage. If, during phenol extraction, the white interphase between the organic and aqueous phases is particularly large (such that it might be difficult to remove the aqueous phase without transferring some of this material), simply perform a second phenol-chloroform extraction before proceeding to the chloroform extraction.
15. The incorporation of radioactive dCTP during the PCR reaction offers superior sensitivity to EtBr or other DNA-staining techniques. In our experience, it is difficult to set up a quantitative PCR analysis of ChIP DNA using ethidium staining, because the number of cycles needed to produce a product in sufficient quantity to be detected is usually too high for the amplification to be quantitative. Use of $\alpha(P^{33}$-dCTP) is convenient because it does not require extensive shielding, but be sure to follow all institutional guideline for use of radioactive materials.
16. Remember that the listed cycling parameters are arbitrary, and will need to be optimized for each experiment.
17. Following PCR, several calculations must be performed to determine the level and specificity of binding of the protein of interest to a specific segment of DNA. To calculate the specific enrichment of the DNA fragment in the IP, calculate the ratio of radioactivity in the target fragment relative to the reference fragment. To adjust for inherent differences in the amplification of these two fragments, nor-

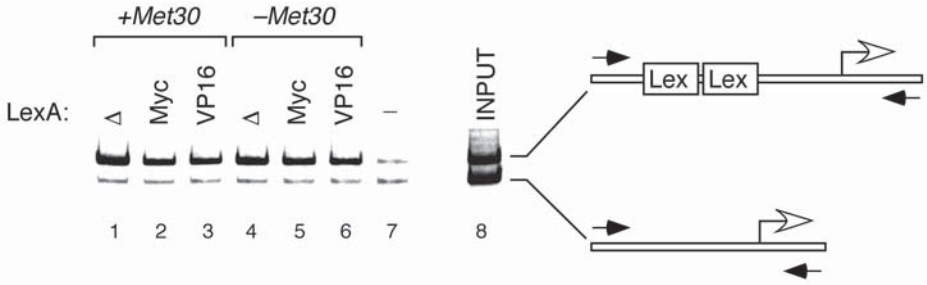

Fig. 2. Binding of LexA-fusion activators to a target gene: standard PCR analysis. In this experiment, the bacterial DNA-binding protein LexA was used, either alone ( ), or fused to the Myc or VP16 activation domains ***(8)***. These chimeric proteins, which were tagged with the HA-epitope tag, were expressed in yeast cells that either possessed a functional version of the VP16 co-activator Met30 *(+Met30*), or in which Met30 had been inactivated (*–Met30*). ChIP was used to demonstrate that all LexA-fusion proteins occupied the LexA target gene, regardless of whether or not *Met30* was present. In this yeast strain, two nearly identical reporter genes were present (*at right*), one of which contained LexA-binding sites, the other of which did not. In this way, the same primers were able to amplify the target (upper) and reference (lower) fragments. The extent of binding of LexA-fusion proteins to the target promoter is thus directly represented by the fold enrichment of the upper band relative to the lower.

malize this ratio to the same ratio calculated from the INPUT DNAs. The resulting number is the fold enrichment of the target fragment. Finally, to determine whether the signal is specific for the particular protein, normalize the fold enrichment of the specific IP with the same number from the control IP, performed either using an irrelevant antibody or, when appropriate, the nonepitope-tagged yeast sample. An example of ChIP DNA analyzed by this method is presented in **Fig. 2**.

18. We strongly recommend analyzing each sample in triplicate.
19. During the exponential phase of amplification, the level of fluorescence corresponds directly to the amount of target DNA sequence present in the reaction. Thus, the cycle number at which each PCR product reaches a particular exponential phase (referred to as the "cycle threshold") is a representation of the relative abundance of each target DNA sequence. The relative amounts of target DNAs can be calculated using the following formula:

$$f = 2^{-(C_1 - C_2)}$$

where f is the fold enrichment of PCR products amplified with primer set 1 compared to primer set 2, and C_1 and C_2 are the cycle thresholds for each primer set. Thus, using this formula, one can easily quantify how much more gene-specific DNA compared to reference DNA is present in ChIP immunoprecipitate:

$$f = 2^{-(C_X^{IP} - C_{\mathrm{Re}f}^{IP})}$$

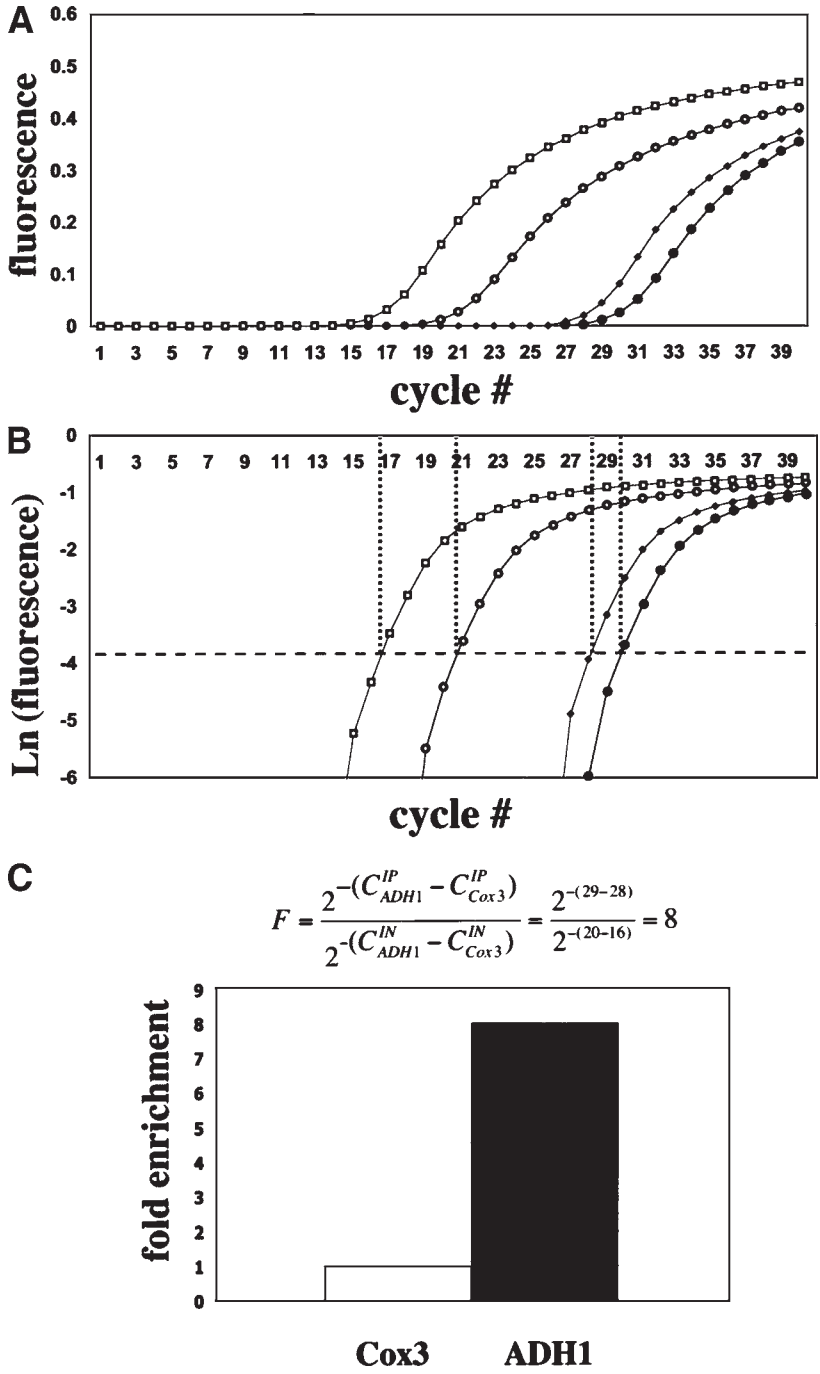

Fig. 3. 19S Proteasome subunit Rpt4 is recruited to the active *ADH1* gene. The ChIP assay was performed with a yeast strain in which the 19S proteasome subunit

This simple equation assumes that the reference and gene-specific fragments are amplified with equal efficiency. Because this is generally not true, it is necessary to calculate the relative amplification efficiency for the two primer sets with INPUT DNA ($C_X^{IN} = C_{\mathrm{Re}f}^{IN}$), and normalize ChIP DNA signals to this value:

$$F = f^{\mathrm{IP}}/f^{\mathrm{IN}}$$

This value, *F*, gives the true fold enrichment of signal from the target protein at the target DNA fragment, relative to the reference DNA sequence. If epitope-tagging was used, this value can be further normalized to the same ratio calculated from experiments performed on untagged control yeast; in this way, the signal intensity can be directly attributed to the presence of the epitope on the specific protein of interest. An example of ChIP DNA analyzed by Q-PCR is presented in **Fig. 3**.

Acknowledgments

We thank Steve Buratowski, Shiv Grewal, Stephen Johnston, and Zhiguo Zhang for help and advice with ChIP assays. Work in the Tansey Laboratory is supported by U.S. Public Health Service grants CA-13106 from the National Cancer Institute and GM067728 from the Institute of General Medical Sciences. EE is an Englehorn Scholar. WPT is a Leukemia and Lymphoma Society of America Scholar.

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Fig. 3. *(continued)* Rpt4 was epitope-tagged with a carboxy-terminal 3xHA epitope tag ***(6)***. After immunoprecipitation with the 12CA5 antibody and reversal of crosslinks, co-precipitating DNAs were detected by Q-PCR. **(A)** Output of real-time thermocycler, showing fluorescence intensities (as a function of cycle number) of amplified products corresponding to the *COX3* reference fragment in the INPUT (□) and IP samples (■) and the target *ADH1* fragment in the INPUT (○) and IP samples (●). **(B)** As in (A), except that log fluorescence intensity value is presented on the *y*-axis. The horizontal dashed line represents the fluorescence value of the cycle threshold (the point at which amplification reactions are quantitative). The cycle number at which each amplification reaction crosses the cycle threshold (vertical dashed lines) is a representation of relative the amount of each DNA present in the reaction, and is used as the value *C* in the calculation presented in **(C)**.

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21

Isolation of Yeast Nuclei and Micrococcal Nuclease Mapping of Nucleosome Positioning

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Summary

Chromatin structure and nucleosome positioning play a crucial role in gene expression regulation. Nucleosome positioning is often inferred by the protection of underlying DNA to nucleases. Because nucleases are excluded by plasma membranes, chromatin mapping requires isolating nuclei from cells and digesting the chromatin *in situ* with nucleases. The quality of this data is highly dependent on the nuclei preparation. Here we describe a method to isolate nuclei from the budding yeast *Saccharomyces cerevisiae* and the use of micrococcal nuclease to map the chromatin structure at the *RNR3* gene. Nuclei isolated by this procedure are competent for many of the common chromatin mapping and detection procedures.

Key Words: Chromatin; nucleosome mapping; nuclei preparation; nucleases; yeast.

1. Introduction

As the packaged form of eukaryotic genetic material, chromatin plays a pivotal role in transcription regulation and the other DNA-related processes ***(1)***. One important aspect of chromatin structure is nucleosome positioning: the preferred location of a nucleosome over a certain region of DNA. A variety of methods have been developed to study nucleosome positioning in vivo ***(2–6)***. Generally, chromatin in either isolated nuclei or permeabilized cells is probed with reagents that preferentially attack nucleosome-free DNA, such as micrococcal nuclease (MNase), DNase I, restriction endonucleases, or chemical reagents like methidiumpropyl–ethylenediaminetetraacetic acid (EDTA) (MPE·Fe[II]). Each method has its advantages and disadvantages. For example, the procedure for isolating nuclei is more time-consuming than that using permeabilized spheroplasts, but usually provides higher-quality mapping data and is essential when reiterative primer extension is used to detect the diges-

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

tion products. After purification of genomic DNA, the digestion products are detected by either indirect end labeling (Southern blotting) or by a reiterative primer extension method using thermostable DNA polymerases *(7)*. The indirect end-labeling procedure is useful for analyzing chromatin structure over a relatively large area, up to 2 kilobase (Kb) pairs, but its resolution limits for most applications are at best approx 20–50 base pairs. On the other hand, primer extension is considered "high resolution" because it can detect changes at the resolution level of a single base pair, but is more fastidious and is prone to artifacts caused by the nicking of DNA during chromatin isolation.

Here we describe a procedure for isolating nuclei from yeast and analyzing nucleosome positioning using micrococcol nuclease combined with the indirect end-labeling method. Nuclei prepared from this method can be used to probe chromatin structure using DNase I and restriction endonucleases as well, and is of high enough quality to use the reiterative primer extension detection method *(7)*. We have used this protocol to study the chromatin structure at genes where nucleosome positioning plays an important role in their regulatory mechanism, most notably the DNA damage-inducible gene *RNR3 (8–10)*.

2. Materials

2.1. Nuclei Isolation and MNase Digestion

1. Sorbitol buffer: 1.4 *M* sorbitol, 40 m*M* HEPES-Na, pH 7.5, 0.5 m*M* $MgCl_2$. Sterilize by filtration and store at 4°C.
2. Sorbitol wash buffer: sorbitol buffer supplemented with polymethylsulfonyl fluoride (PMSF) and beta-mercaptoethanol (BME) immediately before use at 1 m*M* and 10 m*M*, respectively.
3. Sorbitol digestion buffer: sorbitol buffer supplemented with PMSF and BME immediately before use at 1 m*M* and 2 m*M*, respectively.
4. Ficoll buffer: 18% Ficoll 400 (Amersham Biosciences Corporation, Uppsala, Sweden); 20 m*M* PIPES-Na, pH 6.5; 0.5 m*M* $MgCl_2$. Sterilize by filtration and store at 4°C.
5. Glycerol-Ficoll buffer: 7% Ficoll 400, 20% glycerol, 20 m*M* PIPES-Na, pH 6.5, 0.5 m*M* $MgCl_2$. Sterilize by filtration and store at 4°C.
6. Digestion buffer: 10 m*M* HEPES-Na, pH 7.5, 0.5 m*M* $MgCl_2$, 0.05 m*M* $CaCl_2$. Autoclave and store at 4°C.
7. BME: 14.25 *M* stock, store at 4°C.
8. PMSF solution: 200 m*M* PMSF dissolved in absolute ethanol, store at 4°C.
9. Zymolyase: 10 mg/mL Zymolyase 100T (Seikagaku Corporation, Tokyo, Japan) prepared in sorbitol buffer and stored in aliquots at –80°C.
10. Micrococcal nuclease: 10 U/µL micrococcal nuclease (Worthington Biochemical Corporation, Lakewood, NJ) dissolved in water and stored in multiple small aliquots at –20°C. Do not freeze-thaw.

2.2. Genomic DNA Isolation

1. 0.5 *M* EDTA-Na: pH 8.0, autoclave and store at room temperature.
2. DNase-free RNase A: 5 mg/mL, prepared as described in another publication ***(11)***, stored in aliquots at –20°C.
3. 20% sarkosyl: Dissolve N-lauryl sarkosin (free acid) in water, adjust to a pH of 7.5 with NaOH, and sterilize by filtration. Store at room temperature.
4. 5 *M* $NaClO_4$: Dissolved in water and stored at room temperature.
5. Protease K: 10 mg/mL protease K (Sigma-Aldrich Corporation, St. Louis, MO), dissolved in water and stored at –20°C.
6. Phenol-chloroform-isoamyl alcohol: Buffer-saturated phenol:chloroform:isoamyl alcohol mixed at a ratio of 25:24:1 and stored at 4°C.
7. Chloroform-isoamyl alcohol: chloroform:isoamyl alcohol mixed at a ratio of 24:1 and stored at 4°C.
8. 3 *M* sodium acetate: Dissolved in water, adjusted to a pH of 5.2 with glacial acetic acid. Autoclaved and stored at room temperature.
9. Absolute ethanol: pre-chilled at –20°C.
10. 0.1X TE: 1 m*M* Tris-HCl, pH 8.0, 0.1 m*M* EDTA, autoclaved.

2.3. Indirect End-Labeling Detection of MNase Digestion Products

1. 5X TBE: (perL) 54 g Tris base, 27.5 g boric acid, 20 mL 0.5 *M* EDTA, pH 8.0. Filter through a 0.45-µm membrane to retard precipitation.
2. 0.2 *M* HCl: 20 mL concentrated HCl (38%) plus 980 mL water.
3. Denaturing buffer: 1.5 *M* NaCl, 0.5 *M* NaOH.
4. Renaturing buffer: 1.5 *M* NaCl, 1 *M* Tris-HCl, pH 7.4.
5. 20X SSC: dissolve 175.3 g NaCl and 88.2 g sodium citrate into 800 mL of distilled water, bring the volume to 1 L with water, and adjust the pH to 7.0 with a few drops of 10 N NaOH.
6. Prehybridization buffer: 6X SSC, 5X Denhardt's reagent, 0.5% sodium dodecyl sulfate (SDS), 100 µg/mL sheared salmon sperm DNA (ssDNA). Salmon sperm DNA (10 mg/mL stock in water) is denatured by heating at 95–100°C and is added to the prehybridization solution pre-warmed to 60°C. The preparation of 50X Denhardt's reagent and ssDNA is described elsewhere (***11***).
7. Blot washing buffer: 1X SSC, 0.1% SDS.

3. Methods

3.1. Yeast Cell Culture and Harvest

1. Grow a 5-mL culture of yeast in the appropriate culture medium at 30°C on a roller wheel or shaker.
2. The next morning, reseed the saturated culture into a 500-mL flask containing 100 mL of fresh medium. Dilute the culture to an appropriate starting density as to achieve log-phase growth (OD_{600} of about 1.0) by the evening.

3. Reseed a portion of the 100-mL starter culture into two 2-L flasks containing 500 mL of culture medium so that the OD_{600} of the culture will be between 0.8 and 1.2 the next morning. At this step, the inoculation volume is empirically determined and depends on the growth rate of the strain, on temperature, and on medium (*see* **Note 1**).
5. Grow overnight at 30°C until the culture achieves a density of OD_{600} approx 1.0 and collect the cells by centrifugation at 4500*g* using, for example, a Sorvall SLC-6000 rotor (Kendro Laboratory Products, Newtown, CT) for 5 min. Centrifugation can be carried out at 4°C or room temperature. Pour off culture medium and place the cells on ice.
6. Prior to centrifugation, equilibrate a shaking water bath to 30°C and prepare an aliquot of sorbitol wash buffer sufficient for all samples and keep on ice.
7. Resuspend the cell pellet in 30 mL sorbitol wash buffer using a pipet and transfer to a round-bottom 50-mL centrifugation tube (Nalgene Nunc International, Rochester, NY, cat. no. 3110-9500). Collect cells for 5 min at 4500*g* in a Sorvall HB-6 rotor at 4°C. Remove as much wash buffer as possible by aspiration, and keep on ice.
8. Repeat the wash step once more as described in **step 7**.

3.2. Spheroblast Preparation

1. Prepare a fresh aliquot of sorbitol digestion buffer and store on ice.
2. Weigh the pellet (it should be approx 1 g/L of culture at a density of OD_{600} 1), and resuspend it in 4 mL sorbitol digestion buffer per gram (wet weight) of cells using a pipet.
3. Incubate the cell suspension at 30°C for 10 min in the centrifuge tube with gentle shaking, during which thaw the zymolyase in your hand then keep it on ice.
4. Add 1/5 of a volume of a 10 mg/mL zymolyase stock per gram of cell pellet (final enzyme concentration is 0.5 mg/mL), and incubate at 30°C with gentle shaking for 20 min.
5. After 20 min, examine the cell suspension to monitor the extent of digestion. This is achieved by placing 1–2 µL on a glass slide, placing a cover slip over the sample, and examining it under a light microscope. A good zymolyase treatment should convert almost all the cells into spheroblasts. Spheroblast formation is confirmed by hypotonic lysis or squeezing (*see* **Note 2**).
6. Add cold sorbitol digestion buffer to bring the total volume of buffer added to the cell pellet up to 30 mL. For example, if the cell pellet was resuspended into 4 mL in **step 2**, add 26 mL of buffer. Centrifuge at 4500*g* for 5 min at 4°C in a Sorvall HB-6 rotor.
7. Prepare an aliquot of sorbitol buffer supplemented with 1 m*M* PMSF and place on ice. This should be done during the previous centrifugation step. Completely but gently resuspend the pellet in 30 mL of ice-cold sorbitol buffer (with 1 m*M* PMSF) (*see* **Note 3**). Centrifuge at 4500*g* for 5 min at 4°C in an HB-6 rotor. Remove as much buffer as possible by aspiration, and keep on ice.
8. Repeat **step 7**.

3.3. Nuclei Isolation

1. Resuspend the pellet in 20 mL ice-cold Ficoll buffer and transfer to a pre-chilled glass homogenizer (Thomas Scientific, Swedesboro, NJ, cat. no. 3431E25). Homogenize by six to eight smooth and even strokes on ice using a Teflon pestle attached to an electric drill revolving at top speed.
2. Prepare a 20 mL cushion of cold glycerol-Ficoll buffer supplemented with 1 m*M* PMSF in a round-bottom 50-mL tube and place on ice. Gently layer the homogenate onto the top of the cushion. (The two phases should be clearly separated.)
3. Collect the nuclei by centrifugation using an HB-6 rotor at 21,500*g* for 30 min at 4°C. (A swinging bucket rotor, like HB-6, must be used at this step.) Aspirate the supernatant completely.
4. Resuspend the pellet in 20 mL Ficoll buffer (*see* **Note 4**). Then cap the tube tightly and vortex at top speed for 2.5 min, chill on ice for 5 min, and vortex for another 2.5 min for a total of 5 min of vortexing.
5. Centrifuge the sample at 3300*g* in a Sorvall HB-6 rotor for 15 min at 4°C. Gently remove the tubes from the rotor and carefully transfer the supernatant to a fresh 50-mL round-bottom tube using a 10-mL pipet. Avoid the pellet, which will be loose. It is better to leave a small fraction behind than risk transferring some of the pelleted material. This step removes intact cells and large debris.
6. Centrifuge the supernatant from **step 5** at 21,500*g* for 30 min at 4°C in an HB-6 rotor. Aspirate the supernatant thoroughly and place on ice. This is the nuclear pellet.
7. Resuspend the pellet in 10 mL digestion buffer by pipeting (*see* **Note 5**).
8. To estimate the amount of nuclei recovered, dilute 100 µL of the suspension (**step 7**) in 900 µL digestion buffer and measure the OD_{600}. The reading should be around 0.2 for 1 g of wild-type cells (*see* **Note 5**).
9. Recover the nuclei by centrifuging at 21,500*g* for 15 min at 4°C. Aspirate the supernatant and place nuclear pellet on ice.
10. Resuspend the pellet in 2.4 mL digestion buffer, making minor adjustments based on the estimated nuclei density measured in **step 8** (*see* **Notes 5** and **6**).

3.4. Micrococcal Nuclease Digestion

1. Thaw the micrococcal nuclease stock (10 U/µL) on ice and prepare serial dilutions in digestion buffer of 0.8, 0.4, 0.2, and 0.1 U/µL.
2. Divide the nuclei suspension into 6 × 400 µL aliquots in 1.5-mL tubes and prewarm at 37°C for 10 min.
3. Add 4 µL of each concentration of MNase to each of four nuclei aliquots (final enzyme concentration will be 8, 4, 2, and 1 U/mL, respectively). Mix by gently vortexing and incubate at 37°C for 10 min. One of the remaining nuclei aliquots will be used as a zero digestion control, and the other used for preparing genomic DNA that will be digested with MNase after deproteination/purification (naked DNA digestion). The naked DNA sample will be digested later in **Subheading 3.5., step 8**.

4. Add 8 µL 0.5 *M* EDTA (final concentration is 10 m*M*) and mix by vortexing.

3.5. Purification of Genomic DNA

1. Add 8 µL 5 mg/mL RNase A (final concentration is 100 µg/mL) to each tube, vortex, and incubate at 37°C for 2 h.
2. Add 63 µL 20% Sarkosyl (2.5% final), 20 µL 5 *M* $NaClO_4$ (200 m*M* final), and 2.5 µL 10 mg/mL Protease K (50 µg/mL final). Mix by vortexing, and incubate overnight at 55°C.
3. Add 500 µL phenol-chloroform-isoamyl alcohol, mix by vortexing for 2 min, and spin at full speed for 10 min.
4. Carefully transfer the supernatant to fresh tubes, add 8 µL 5 mg/mL RNase A, mix well, and incubate at 37°C for 30 min.
5. Repeat the phenol:chloroform:isoamyl alcohol extraction once, and then extract once with chloroform:isoamyl alcohol.
6. Carefully transfer a fixed amount of the supernatant (300–400 µL) to new tubes, and add 1/10 volume of 3 *M* sodium acetate and 2 volumes of cold absolute ethanol. Mix and place on dry ice for 30 min. Precipitate the DNA by centrifugation at high speed, aspirate the supernatant, wash the pellet with 1 mL 70% ethanol, and air-dry.
7. Dissolve each DNA pellet in 100 µL 0.1X TE except for the naked DNA sample (*see* **step 8**). Optional: verify the recovery of DNA by spectrometry. Expect to recover approx 50 µg of DNA from each sample.
8. To prepare a naked DNA digest sample, dissolve the DNA pellet in 400 µL Digestion buffer, split into two 200-µL aliquots, and digest with 2 and 1 U/mL MNase for 10 min at 37°C (*see* **Note 7**). Add 3 µL 0.5 *M* EDTA to stop the reaction, and isolate the DNA as described in **steps 5** and **6**. Dissolve the pellet in 50 µL 0.1X TE.
9. Analyze 2 µL of each DNA sample (both nuclei and naked DNA) on a 1.6% agarose gel. A good preparation of nuclei DNA should yield >5 nucleosomal bands. *See* **Fig. 1** for an example.

3.6. Detection of Digestion Products by Indirect End-Labeling

1. Digest 10 µL of each DNA sample (about 5 µg) with the proper restriction enzyme overnight at 37°C (*see* **Notes 8** and **9**). We typically perform our restriction endonuclease digestions in a 100 µL volume using 20–25 U of enzyme.
2. Precipitate the DNA by adding 1/10 of a volume of 3 *M* sodium acetate and 2 volumes of absolute ethanol, incubating on dry ice for 20 min and centrifuging the samples at high speed. Air dry the samples and dissolve the DNA in 25 µL of 0.1X TE. Add 5 µL 6X agarose gel loading buffer containing bromophenol blue dye *(11)* and load onto a 1.4% agarose gel prepared in 1X TBE. We typically use 27-cm long gels for good resolution.
3. Run the gel in 1X TBE buffer for 4 h at 5.5 V/cm.
4. Trim the gel by cutting about 2 cm below the bromophenol blue dye and 2 cm below the loading wells.

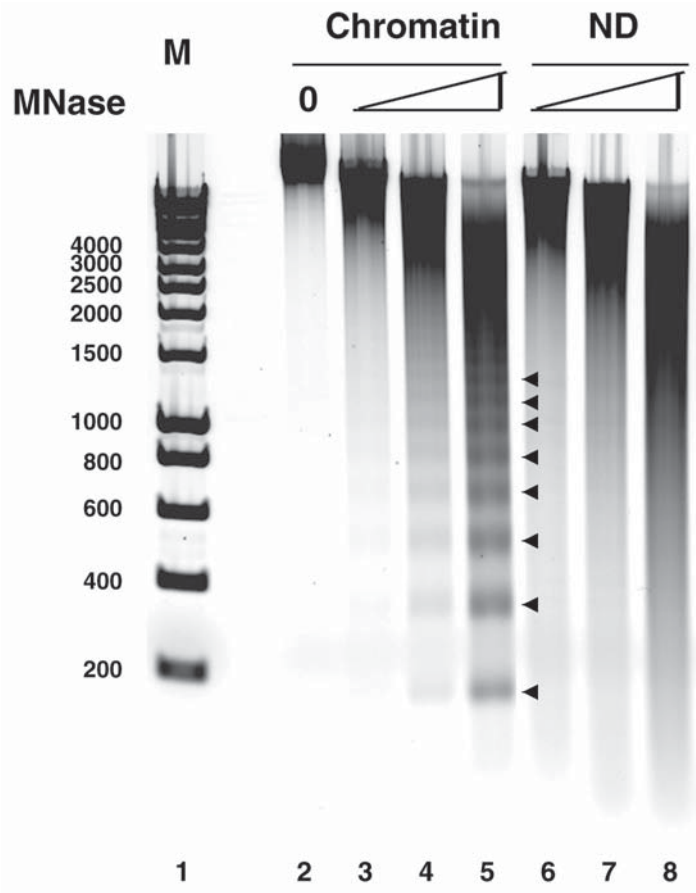

Fig. 1. Agarose gel electrophoresis of micrococcal nuclease (MNase) digestion of nuclei and naked DNA. Lane 1 (M) contains a molecular marker with the corresponding length of some bands labeled on the left (in base pairs). Nuclei isolated from wild-type yeast strain (PH499) were digested with 0, 2, 4, and 8 U/mL MNase, respectively (lanes 2–5). "Naked DNA" (ND, lanes 6–8) is purified genomic DNA digested with 0.5, 1, and 2 U/mL MNase, respectively. The DNA was separated on an 1.6% agarose gel, and was stained with 0.5 µg/mL ethidium bromide. Filled triangles correspond to mono-, di-, tri-, and oligo nucleosomal DNA "ladder" observed in DNA from MNase digested chromatin.

5. To aid in the transfer of the larger DNA fragments, soak the gel in 0.2 *M* HCl for 10 min with gentle shaking.
6. Rinse the gel with deionized water, transfer to denaturing buffer, and incubate for 45 min with gentle shaking.

7. Rinse the gel with deionized water, transfer to re-naturing buffer, and incubate for 20 min with gentle shaking. Replace the renaturing buffer and continue for an additional 25 min.
8. Transfer DNA to a charged nylon membrane using the capillary-transfer method *(**11**)*.
9. UV cross-link the DNA to the membrane (120,000 μJ/cm^2), and then wash the membrane with blot-washing buffer (1X SSC, 0.1% SDS) at 65°C for about 30 min to remove the loading dye(s) and small pieces of agarose from the gel. The membrane is ready for prehybridization or can be dried and stored at room temperature.
10. Transfer the membrane from blot-washing buffer to prehybridization buffer prewarmed to 60–65°C. Prehybridize for at least 4 h with gentle shaking.
11. Add a body-labeled probe to a concentration of at least 100,000 cpm/mL of specific radioactivity, and continue the incubation at 60–65°C overnight (*see* **Notes 9** and **10**).
12. Wash the membrane with blot washing buffer twice (15 min each) at 60–65°C, and twice (15 min each) at room temperature. Mount the membrane onto a smooth and clean surface, such as a used X-ray film, cover with plastic wrap, and expose to X-ray film or phosphor imager screen (Molecular Dynamics Incorporation, Sunnyvale, CA). *See* **Fig. 2** for an example result.

4. Notes

1. The growth rate of each strain should be calculated ahead of time. You can estimate the growth rate during the incubation at **Subheading 3.1., step 2**. For some strains with a severe slow-growth phenotype, it might be necessary to grow the 100-mL starter culture overnight in order to get enough cells for the inoculation of the 500-mL preparative cultures at **Subheading 3.1., step 3**. Because certain mutant strains are inconsistent in recovering from stationary phase, we recommend seeding from a starter culture that is in log phase rather than seeding directly from a saturated culture.
2. The spheroblasts are mostly oval in shape, and have rougher edges and more irregular light diffraction under phase-contrast microscopy compared to intact cells. To verify the extent of spheroblast formation, squeeze the spheroblasts by pushing the cover glass against the slide and move back and forth several times. After squeezing, the oval spheroblasts will become thin rods of various lengths. Intact cells will retain their shape. Alternatively, add a drop of water to the edge of cover glass and look for the rupture of the spheroblasts into ash-like "ghosts." Too long of an incubation with zymolyase at 30°C should be avoided.

Fig. 2. *(opposite page)* Chromatin mapping of *RNR3* by micrococcal nuclease (MNase) digestion and indirect end labeling**.** Nuclei were prepared from yeast cells (PH499) grown in YPD and treated with (+ MMS) or without (–MMS) 0.03% MMS for 2 h. Nuclei were digested with 0, 4, and 8 U/mL of micrococcal nuclease (MNase) for 10 min at 37°C. Naked DNA (ND) was digested with 0.5 and 1 U/mL of MNase. The DNA was purified and analyzed as described in **Subheading 3.5.** and was digested to completion with *Pst*I restriction endonuclease, which cuts at +731 (translation start site as +1) of the *RNR3* gene.

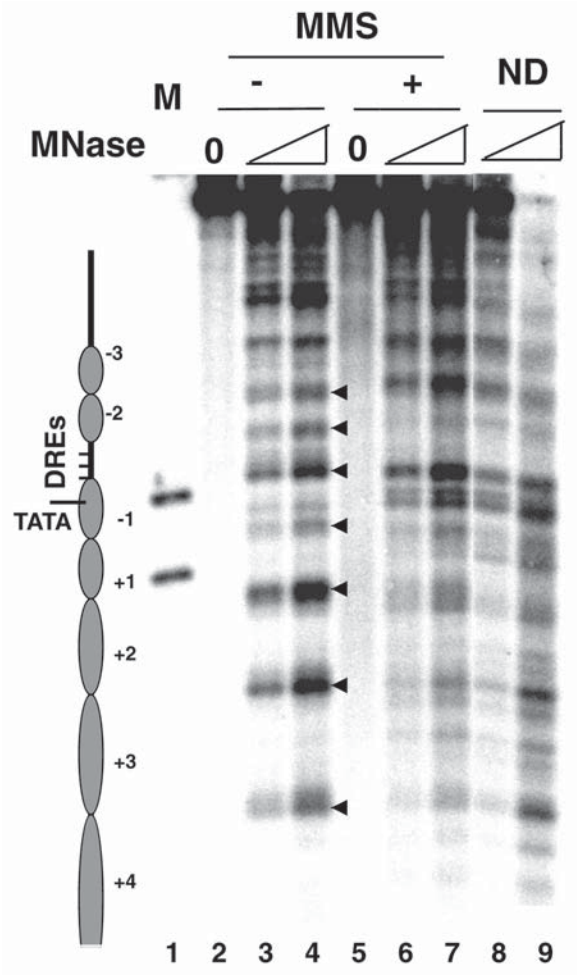

Fig. 2. *(continued)* The products were separated on an 1.4% agarose gel prepared in 1X TBE buffer, transferred to Nylon membrane, and detected by a radioactively labeled probe corresponding to (+486 to +725) of *RNR3*. Lanes 2–7 are chromatin samples. Lane 8 and 9 are the naked DNA samples. 0 represents the samples not treated with MNase. The filled triangles represent the internucleosomal hypersensitive sites in the wild-type chromatin under the repressed state (–MMS). Note that the hypersensitive sites are lost or diminished and the regions protected from MNase digestion were exposed in the chromatin samples of MMS-treated cells, suggesting the loss of nucleosome positioning. A schematic of the *RNR3* gene and the locations of the DNA Damage Responsive Elements (DREs) and TATA box are also indicated on the left. M is a marker prepared from genomic DNA digested with *Pst*I in combination with *Eag*I (cut at +8) and *Mlu*I (cut at –186), respectively.

3. Spheroblasts are fragile and care should be taken during pipeting and resuspending to avoid extensive breakage. We recommend that the pellet be gently resuspended in 5 mL buffer first, then slowly add the remaining 25 mL of buffer. Mix gently by inverting the capped centrifuge tube several times.
4. It is important to completely resuspend the pellet before vortexing. We recommend first resuspending the pellet in 5 mL buffer using a pipet, then adding the remaining 15 mL of buffer.
5. It is important that the nuclei density, and hence the amount of DNA, be as close as possible among all samples. A convenient way to achieve this is to measure the optical density (OD_{600}) of each nuclei sample at **Subheading 3.3., step 7**. Using this value, resuspend each of the nuclear pellets in the appropriate volumes in **Subheading 3.7., step 10** to achieve an equal nuclei density among all samples in the final digestion reaction. Estimating nuclei density by this method is effective in most cases. However, for some mutants or growth conditions additional adjustments in the amount of nuclei or MNase concentrations should be made based on experience. For example, nuclei preparations from some mutants (such as *tup1* or some temperature-sensitive mutants exposed to 37°C) and cells treated with DNA-damaging agents yield less (up to 50%) DNA per OD_{600} of nuclear suspension compared to wild-type cells.
6. The digestion buffer described in this protocol works well for DNAse I and MNase mapping. If chromatin structure is being probed by the restriction endonuclease accessibility assay ***(5)***, resuspend the final nuclear pellet (**Subheading 3.3., step 10**) in 10 m*M* Tris-HCl, pH 7.4, 50 m*M* NaCl, 10 m*M* $MgCl_2$, 0.5 m*M* spermidine, 0.15 m*M* spermine, 0.2 m*M* EDTA, 0.2 m*M* EGTA, and 5 m*M* BME.
7. The efficiency of MNase digestion may vary significantly among different naked DNA samples. It is highly recommended to do a test digestion first to find the proper MNase concentration for each sample, and then carry out the digestion on a larger scale.
8. The total amount of DNA digested in each sample should be as close as possible. Adjusting the DNA quantities among all samples can be achieved by using gel-scanning software to quantify the amount of DNA in the undigested ("0" MNase) sample, which appears as a thick band in the gel at **Subheading 3.5., step 9**. Alternatively, DNA concentration can be measured by spectrometry as mentioned in **Subheading 3.5., step 7**.
9. Choosing a restriction enzyme and probe to use in indirect end-labeling experiments depends on the availability of restriction sites around the chromatin region of interest. Usually the restriction site should be about 300–2000 base pairs away from the region of interest. We typically prepared PCR-generated probes of about 200 base pairs in length.
10. Body-labeled probes are prepared using any commercially available random primer labeling system.

Acknowledgments

We gratefully acknowledge our colleague Dr. Robert T. Simpson for all of his assistance in teaching us how to map chromatin structure in yeast. Portions of the protocol described here originated in his laboratory. We also thank Drs. Mai Xu and Bing Li for valuable suggestions. This work was supported by funds provided by the National Institutes of Health (GM58672) and by an Established Investigator Grant from the American Heart Association to J.C.R.

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22

Study of Transcriptional Regulation Using a Reporter Gene Assay

Yu Fu and Wei Xiao

Summary

Study of gene expression can be facilitated by using a reporter gene assay. Instead of directly measuring the level of target gene mRNA, one can clone the promoter region of the gene of interest in front of a reporter gene and measure the reporter gene expression as a reflection of the expression of the gene of interest. We describe a simple *lacZ*-fusion system to measure the activity of the reporter gene product β-galactosidase. Different strategies of making the fusion construct and their applications are also discussed. This method is particularly useful to dissect the promoter region of the gene of interest and is also used in other experimental protocols such as the yeast two-hybrid analysis.

Key Words: Yeast; β-galactosidase assay; transcription; gene expression; method; reporter.

1. Introduction

The conventional analysis of transcriptional regulation of gene expression is to measure the steady-state transcript level by methods such as Northern hybridization, reverse-transcriptase polymerase chain reaction (RT-PCR) and more recently real-time RT-PCR. The study of gene expression has been greatly facilitated by using a reporter gene system, in which a readily assayed gene (reporter gene) product is produced under the control of promoter element(s) of your favorite gene (*YFG*) under investigation ***(1)***. In most cases, the entire promoter region of *YFG* or a specific regulatory element under investigation, such as an upstream activating sequence (UAS), upstream repressing sequence (URS), enhancer, or silencer, is cloned into a reporter vector at the multiple cloning site (MCS). The confirmed recombinant plasmid is then introduced into the yeast host cells and the quantification of reporter product indirectly provides information on the transcription activity of the promoter or element

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

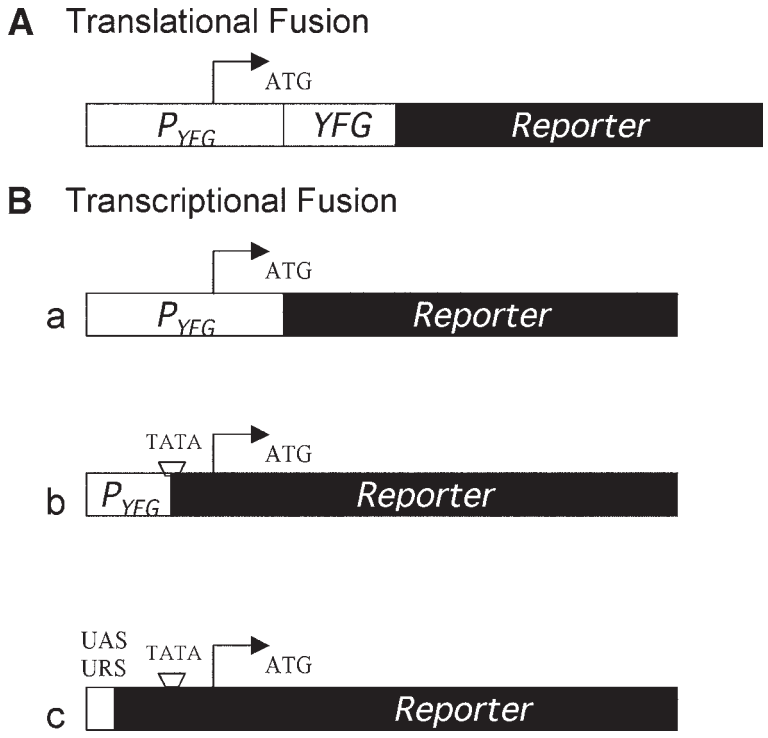

Fig. 1. Different reporter fusion constructs. **(A)** A diagram of translational fusion. **(B)** Three possible scenarios of transcriptional fusions. (a) The reporter gene provides the entire open reading frame and all the promoter region is from *YFG*. (b) The reporter gene provides basic transriptional elements such as TATA box for RNA PolII assembly and the transcriptional initiation site, and the entire regulatory component is from *YFG*. (c) Only a specific regulatory element such as UAS or URS from *YFG* is cloned into the promoter region of the reporter gene to study its specific role(s). Note that the promoter and reporter provided by the vector are from different sources. One good example is the *CYC1-lacZ* fusion construct pLG669Z ***(1)***, which can be directly used to study the regulatory element of *DDI1* ***(8)***. Open box, YFG; filled box, the reporter gene; arrow, transcription initiation site; ATG, translation initiation codon; TATA, the TATA box.

under investigation. The reporter gene can be fused to the regulatory component of *YFG* by either transcriptional or translational fusion ***(2)***. A translational fusion (*see* **Fig. 1A**) requires the in-frame fusion of a portion of coding region of *YFG* with the reporter coding sequence (lacking its own translational initiation codon) so that all the regulatory components are expected to come from *YFG*. This construct is ideal for the initial study of the entire promoter of *YFG*.

On the other hand, a transcriptional fusion (*see* **Fig. 1B**) is constructed by replacing the entire coding sequence of the target gene with that of the reporter gene; the reporter gene has its own translational start (ATG) and stop (TAA) codons and in some cases basic transcriptional elements such as a TATA box. This construct is suitable for the study of specific regulatory elements.

So far, several reporter systems have been developed, including genes encoding chloramphenicol acetyltransferase (CAT), β-galactosidase (β-gal), green fluorescent protein (GFP) and luciferase (Luc), among which the β-gal system ***(3)*** is probably the most popular system used in the budding yeast *Saccharomyces cerevisiae* owing to its stability, reliability, and ease of assay. The prokaryotic β-gal catalyzes the hydrolysis of β-galactosides (e.g., lactose). Because *S. cerevisiae* normally lacks β-gal activity, it is of great benefit to use this system as a reporter in budding yeast. The discovery that the *Escherichia coli lacZ* gene can be fused with yeast genes to produce functional β-gal ***(1,4)*** resulted in the adoption of this system in *S. cerevisiae*.

In this chapter, we describe a procedure for measuring the expression of a gene by fusing its promoter to a *lacZ* reporter. Once initial experiments are performed to establish the correlation between the promoter-reporter fusion construct expression and the endogenous transcript level under various conditions (*see* **Note 1**), the promoter region of *YFG* can be dissected to define *cis*-acting regulatory elements. The reporter gene assay can also be used to monitor environmental stress, detect carcinogens ***(5)***, and to quantitate yeast two-hybrid results ***(6)***. We have used this protocol to study a yeast dual-promoter controlling both *MAG1* ***(7)*** and *DDI1* ***(8)*** genes and illustrate in this protocol the identification of two functionally opposite regulatory elements, UAS and URS ***(8)***.

2. Materials

2.1. Strain

DBY747 (*MAT**a** his3- 1 leu2-3,112 trp1-289 ura3-52*) or any other *S. cerevisiae* strain with appropriate markers.

2.2. Plasmid

YEp353: the plasmid contains a multiple cloning site followed by the *lacZ* gene. It can be amplified in *E. coli* using ampicillin resistance for plasmid selection. It contains the 2 μm origin, FRT and STB for plasmid replication, site-specific recombination, and stability in yeast cells, respectively. In addition, it contains the *URA3* gene for plasmid selection in yeast ***(9)***. A series of plasmids similar to YEp353 but differing in the selectable marker, MCS, and fusion open reading frames (ORFs) are available ***(9)***. If necessary, other single-copy plasmid vectors may be used instead of multicopy plasmids (*see* **Note 2**).

2.3. Medium

1. YEPD medium: 2% Bacto-peptone, 1% Bacto-yeast extract, 2% glucose.
2. SD-Ura: 0.67% yeast nitrogen base without amino acids, 2% glucose, and addition of necessary auxotrophic supplements as described ***(10)***.
   YEPD and SD-Ura plates: to make plates, 2% agar was added to either YEPD or SD medium prior to autoclaving.

2.4. Chemicals and Solutions

1. Buffer Z: 60 m*M* Na_2HPO_4, 40 m*M* NaH_2PO_4, 10 m*M* KCl, 1 m*M* $MgSO_4$, 40 m*M* β-mercaptoethanol, pH 7.0.
2. 0.1% sodium dodecyl sulfate (SDS).
3. Chloroform.
4. 4 mg/mL ortho-nitrophenyl-β-galactoside (ONPG).
5. 1 *M* Na_2CO_3.

3. Methods

3.1. Transforming Yeast Cells With Reporter Constructs

1. Clone the promoter of *YFG* into vector of your choice to make a fusion construct with *lacZ*. In our example, we cloned the *DDI1* promoter and its mutant derivatives into vector YEp353 to make YEp-DDI1-lacZ, YEp-DDI1(-UAS)-lacZ, and YEp-DDI1(-URS)-lacZ. If it is a translational fusion, make sure that the *lacZ* coding sequence (lacking the translation initiation codon) is fused in-frame to the *YFG* coding region (*see* **Fig. 1A**).
2. Transform yeast cells with the reporting constructs. The yeast transformation was performed by using a LiAc method as described ***(11)***. A detailed yeast transformation protocol is also available in this book.
3. After a 3-d incubation at 30°C, pick about 10 individual colonies and streak them on a fresh SD-Ura plate and incubate at 30°C for 2 d.

3.2. β-Gal Liquid Assay

1. Pick up several clones from the fresh streak on the SD-Ura plate (*see* **Note 3**) and grow yeast cells at 30°C in 2 mL liquid SD-Ura with shaking (about 180 rpm) overnight (*see* **Note 4**).
2. 0.5 mL of the overnight culture of yeast cells is used to inoculate 2.5 mL fresh SD-Ura medium; this culture is incubated for another 2 h. Normally the cell density will reach optical density (OD) at 600 nm = 0.2–0.3 (*see* **Note 5**).
3. If the yeast cells need to be treated with a chemical or reagent, add it into the culture tubes as described in **step 2** to the predetermined final concentration. If it is a nonliquid chemical, dissolve the chemical to be tested in an appropriate solvent at the desired concentration prior to use.
4. Continue incubation for the given time period (*see* **Note 1**). This often takes 2–4 h. If it is a time-course study, withdraw 3 mL of culture from a large sample at the given time interval.

5. Take out 1 mL of cells and determine the OD at 600 nm.
6. Collect the remaining 2 mL of cells by centrifugation at 2300*g* for 6 min.
7. Discard the supernatant and resuspend the cell pellet in 1 mL buffer Z.
8. Permeablilize the cells by adding 50 µL of 0.1% SDS, 50 µL of chloroform, and vortexing at top speed for 10 s.
9. Add 200 µl of 4 mg/mL ONPG and gently shake the culture tube at 30°C for 20 min.
10. After the incubation, the reaction is stopped by adding 0.5 mL of 1 *M* Na_2CO_3. The tubes are centrifuged at 2300*g* for 5 min.
11. Transfer 1 mL of supernatant into a cuvet and determine the OD at 420 nm.

3.3. Calculate β-Gal Activity

The β-gal activity is determined through the following equation:

$$\text{Specific activity} = (1000 \times OD_{420nm})/$$
$$[\text{Reaction time (min)} \times \text{Culture volume (mL)} \times OD_{600\ nm}]$$

In this protocol, the reaction time is 20 min; the culture volume is the amount of culture used in the assay, namely 2 mL. The β-gal activity is expressed in Miller units (MU) ***(3)*** (*see* **Fig. 2** for a sample result). Multiple independent experiments are performed for statistic analyses (*see* **Note 6**).

4. Notes

1. In order to use a reporter system to study gene expression, it is very important to establish a correlation between reporter fusion activity and the cognate mRNA level. One such example is given in **Fig. 3**. It is noted that time required to reach maximum induction for mRNA is shorter than that for β-gal activity, which is probably true for most yeast genes. Hence, an appropriate treatment time is to be established through this experiment.
2. The YEp reporter vector system ***(9)*** described in this protocol is based on a 2 µm multicopy plasmid. Based on several genes studied in our laboratory, the reporter constructs faithfully reflect the expression of the genes of interest. If the multicopy plasmid causes a concern, one can use either a centromere-based YCp vector for a single-copy plasmid or a YIp-based plasmid to integrate the reporter gene directly into the locus of *YFG* (***12***).
3. Best results are obtained with freshly streaked yeast cells. Cells taken from frozen stock or an old plate may affect reproducibility in the assay. We perform the β-gal assay within 2 wk after transformation.
4. The β-gal assay was performed with several independent transformants from the same transformation to avoid internal inconsistence.
5. In order to reduce background β-gal assay, an appropriate zero reference is required when measuring OD values, especially when the β-gal activity is low (e.g., under uninduced conditions). To measure cell titer at $OD_{600\ nm}$, the identical culture medium is used to set a reference. To measure β-gal activity at OD_{420nm}, a parallel experiment using cells transformed with the corresponding empty vector is desired to set a reference.

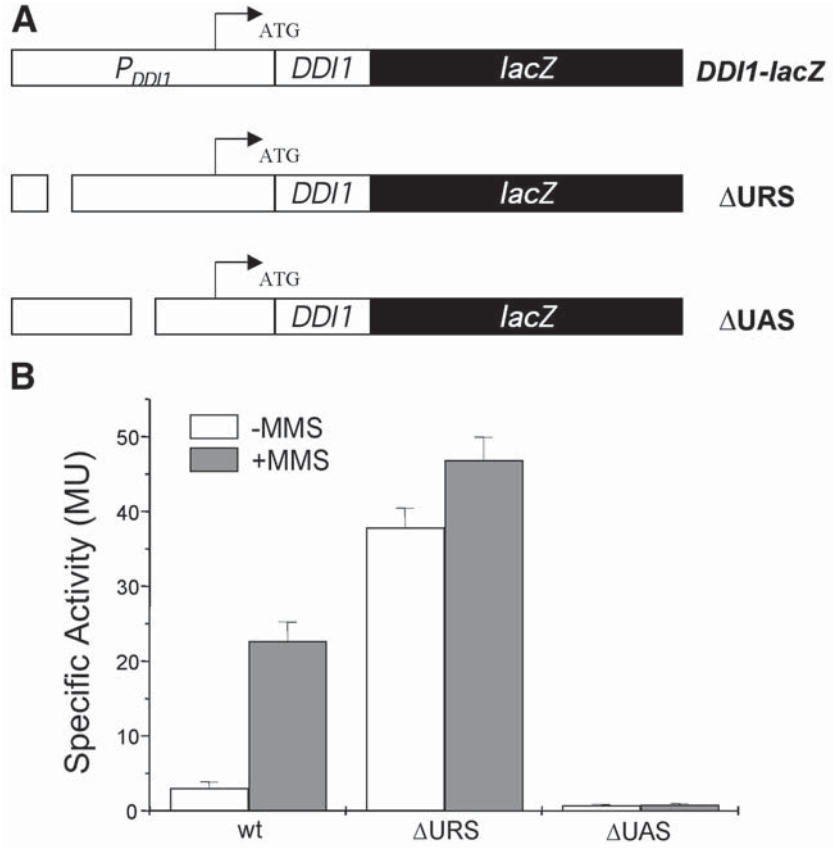

Fig. 2. A typical example of using the reporter gene assay to identify UAS and URS in the promoter of the DNA damage-inducible gene *DDI1*. The results are based on **ref. *8***. **(A)** A schematic diagram of *DDI1-lacZ* translational fusion construct and its internal deletion derivatives. **(B)** The β-gal assay results of yeast transformants of *DDI1-lacZ* construct and its derivatives with or without DNA-damage treatment using methyl methanesulfonate (MMS) as a DNA-damaging agent. The URS element is defined when its deletion or inactivation results in an increased basal-level expression. The UAS element is defined when its deletion or inactivation results in a decreased basal-level expression and/or loss of DNA damage induction. The β-gal activity shown is an average of three independent experiments with standard deviations shown as error bars.

6. Results from various transformants/treatments presented for comparison (e.g., treated vs untreated, full-length promoter vs its derivatives) were always from the same experiment to avoid inter-experimental variations. All the results should take the average of at least three independent experiments.

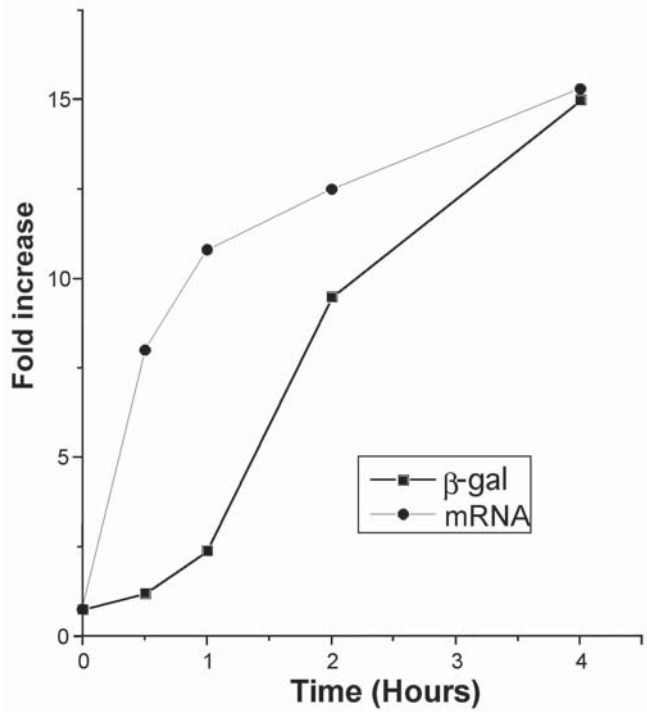

Fig. 3. A typical example of establishing a correlation between *MAG1-lacZ* activity and the *MAG1* steady-state mRNA level under DNA damage conditions. The results are adapted from **ref.** *7*. DBY747 cells were treated with 0.05% MMS for the time as indicated. The relative mRNA level was determined by Northern hybridization band intensity and normalized against the *ACT1* mRNA level as an internal control.

Acknowledgments

The authors wish to thank Michelle Hanna for proofreading the manuscript. This work is supported by the Natural Sciences and Engineering Research Council of Canada operating grant 038338-01 to W.X.

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23

Assessing Telomeric Phenotypes

Catherine LeBel, Michel Larrivée, Amadou Bah, Nancy Laterreur, Nancy Lévesque, and Raymund J. Wellinger

Summary

The concept of telomeres as being the end-part of eukaryotic chromosomes was first described by H. J. Muller and B. McClintock ***(1,2)***. Their pioneering work opened the path for multiple new researches and assays on a thrilling subject, with implications for various domains such as aging, replication, immortality, and cancer. Yeast has been a model of choice to study telomere length, senescence, telomerase activity, telomere cloning, and sequencing with important new techniques being discovered in this species and adapted afterward for other organisms. The main functions of telomeres include the protection of the genome from deletions, recombination, and degradation, and they are therefore essential for genome stability. Their maintenance is assured by a specific enzyme (telomerase) and it is of vital interest for the organism to maintain their length and specific structure. Multiple assays have been described to analyze telomere length and for yeast, Southern blot analysis of terminal restriction fragments (TRFs) remains one of the most popular ones to get a global picture of the state of telomeres in a given experimental setting. However, growth phenotypes (senescence) and fine-structure analyses of the chromosome terminal DNA are also becoming increasingly important. Therefore, the assays that determine those parameters are of highest interest when assessing telomeric phenotypes.

Key Words: Telomere; Southern blot; telomerase; telomere length and structure; X and Y' telomeres; G-tails; in-gel hybridization.

1. Introduction

1.1. Telomere Length Assay

Telomeres are the physical ends of eukaryotic chromosomes and are composed of specific DNA repeat sequences and proteins that can bind to them. The main function of telomeres is to protect the genome from chromosome fusions, deletions, recombination, and degradation, and they are therefore

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

essential for genome stability. In yeast, telomeres of a typical wild-type strain consist of about 300 ± 75 bp of $C_{2-3}A(CA)_{1-6}/(TG)_{1-6}TG_{2-3}$, which is commonly abbreviated $C_{1-3}A/TG_{1-3}$ ***(3–5)***. This terminal repeat tract is necessary and sufficient for all telomeric functions assessed today and when assaying telomeric phenotypes, it is this tract that is the subject of investigations. Thus, for the purpose of this chapter, telomere length refers to the length of this tract only and does not include telomere-associated elements. The length of this track is subject to changes, either increasing or decreasing in length depending on the experimental setting. It is thought that the final average length of telomeric DNA reflects a balance between lengthening and shortening processes and can be very dynamic.

In addition to the $C_{1-3}A/TG_{1-3}$ repeats located at the very end of the chromosomes, yeasts also carry middle repetitive DNA sequences called telomere-associated (TA) sequences (*see* **Fig. 1A**). Virtually all of common yeast laboratory strains carry at least one Y'-element on about two-thirds of all telomeres ***(6)***. When present, this element is usually proximal to the terminal $C_{1-3}A/TG_{1-3}$ repeats and there are two size variants of either 6.7 kb or 5.2 kb (Y' long and Y' short, respectively). These Y'-elements can move in a random fashion during meiosis and/or mitosis, i.e., telomeres without a Y' can gain one, telomeres that had a Y' can lose it, or the elements can become tandemly repeated ***(7)***. Each Y'-element bears a single site for the restriction enzyme *Xho*I about 875 bp proximal from the transition of Y' to telomeric repeats and, for historic reasons, it is this enzyme that is most widely used for an analysis of the most common terminal restriction fragments (TRFs). However, unique sites for other enzymes also occur in the Y'-element and thus liberate TRFs of different lengths for an analysis ***(8)***.

A second class of TA-sequences is called X ***(6)***. This element can vary greatly in size (from 0.3–3.75 kb) and is usually located internally to the Y'-elements on a given telomere. However, on telomeres lacking Y'-elements, it is located immediately after the $C_{1-3}A/TG_{1-3}$ repeats. Both Y'- and X-elements encompass an autonomously replicating sequence (ARS) ***(6)***, but these sequences do not seem essential for establishment or maintenance of telomeres ***(9)***.

Fig. 1. *(opposite page)* Yeast telomere structure and Southern blot of yeast telomeric DNA using different probes. **(A)** Schematic representation of Y'- and non-Y'-telomeres. Most distal on each yeast telomere are approx 300 ± 75 base pairs of $C_{1-3}A/TG_{1-3}$ repeat sequences (represented by a zigzag line). At the very end, the G-rich strand is about 10 to 15 nucleotides longer than the C-rich strand (G-tails). About two-thirds of the telomeres harbor one or more copies of the Y'-element immediately adjacent to those terminal $C_{1-3}A/TG_{1-3}$ repeats (Y'-telomeres). Known sites of *Xho*I cleavage in Y'-elements are shown, and *Xho*I sites at uncertain positions in genomic DNA are in

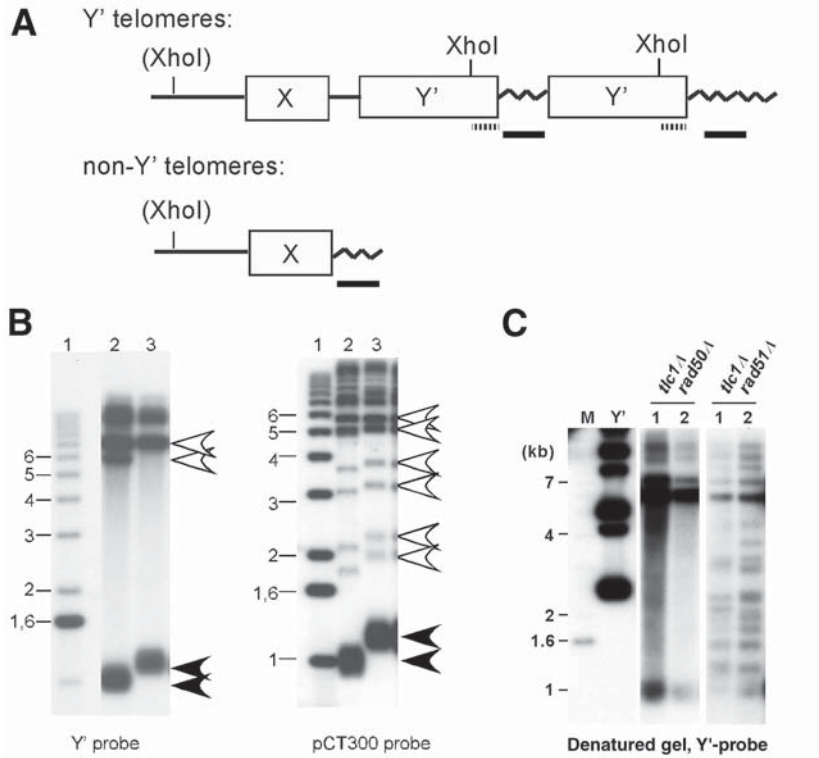

Fig. 1. *(continued)* parentheses. The terminal restriction fragments (TRFs) generated from the Y'-telomeres by the *Xho*I restriction enzyme are about 1.2 kb long. Non-Y'-telomeres bear only an X element yielding TRFs of various sizes, owing to the incertainty of the position of the *Xho*I site in genomic DNA. Hybridization sites to the Y'-probe are shown in blue and hybridization sites to the telomeric probe pCT300 (*see* **Table 1**) are shown in orange. **(B)** Southern blot of yeast genomic DNA from wt and *yku70Δ* cells. Left panel, Hybridization to Y'probe. Lane 1, DNA molecular-weight marker; lane 2, genomic DNA from *yku70Δ* strain; lane 3, genomic DNA from wt strain. Hybridization of the Y' probe to the terminal restriction fragments (TRFs) is shown by black arrows (1.0 kb for *yku70Δ* and 1.2 kb for wt) and two full-length internal Y'-elements are indicated by open arrowheads. Right panel, hybridization to the pCT300 probe. Lane 1, molecular-weight marker; lane 2, genomic DNA of *yku70Δ* strain; lane 3, genomic DNA of wt strain. Hybridization of the pCT300 probe to Y'-TRFs is shown by black arrows (1.0 kb for *yku70Δ* and 1.2 kb for wt) and the hybridization to non-Y' TRFs is shown by open arrowheads. **(C)** Patterns of TRFs occurring in type I and type II survivors. Genomic DNA of two different clones for each survivor type was digested with *Xho*I and hybridized to a Y'-probe. Type II survivors (*tlc1Δrad51Δ*) show amplification of the TG_{1-3} telomeric repeat DNA, whereas type I survivors (*tlc1Δrad50Δ*) show amplifications of the Y'-elements. Molecular-weight marker is shown on left, a control for Y' hybridization in lane 2.

Table 1
Plasmids Used As Probes and Controls

Name	Original plasmid	Fragment liberated with	Description	Ref.
pCT300	pYLPV	*Eco*RI	286 bp of yeast telomeric repeats	***(15)***
pVZY'K	pVZ1	*Kpn*I	600 bp of Y' sequence	***(8,51)***
pMW55	pRS303	*Eco*RV	55 bp of $C_{1-3}A/TG_{1-3}$ repeats	***(17,52)***
pCA75	pVZ1	*Bam*HI	72 bp of $C_{1-3}A$ repeats, used as ssCA	***(15,51)***
pGT75	pVZ1	*Bam*HI	72 bp of TG_{1-3} repeats, used as ssGT	***(15,51)***
CA-probe	Oligo	—	5'-CCCACCACACACACCCACACCC-3'	***(17)***
GT-probe	Oligo	—	5'-GGGTGTGGGTGTGTGTGGTGGG-3'	***(17)***

The Southern blot technique is simple, fast, and useful in the analysis of telomere length. In most cases, the technique relies on the fact that 60–70% of yeast telomeres contain at least one copy of the Y' element with its unique sites for a restriction enzyme (typically *Xho*I). Genomic DNA is extracted from yeast cultures and digested by *Xho*I (release the TRF). It is then migrated on an agarose gel, transferred to a nylon membrane, and hybridized to a telomere-specific probe. Typically, the average length of the TRFs detected for wild-type yeast strain is approx 1.2 kb (875 bp of Y' sequences plus the approx 300 bp of telomeric repeats). This easy and straightforward procedure therefore allows an assessment of telomere length in the vast majority of yeast strains and experimental settings.

1.2. Yeast Senescence Assays

Telomere length is maintained by the *de novo* addition of telomeric repeats by telomerase, yet recombination can also elongate telomeres in the absence of telomerase. When any of the yeast genes essential for the telomerase pathway are deleted, the lengths of the terminal telomeric repeat tracts gradually shorten, chromosome loss rates increase, and most cells enter a terminal growth arrest after 50–100 generations (*see* **Fig. 2A** and **ref. *10***). However, gene conversion and/or recombination mediated by the Rad52 pathway allow telomere lengthening in rare, spontaneous survivor cells ***(11–14)***. These survivors have rearranged and amplified telomeric regions and are categorized into two distinct classes ***(11–14)***. Type I survivors display tandem amplifications of Y'-elements followed by a very short terminal tract of $C_{1-3}A/TG_{1-3}$ DNA; type II survivors display telomeres with very long and heterogeneous-length tracts of $C_{1-3}A/TG_{1-3}$ (*see* **Fig. 2B** and **ref. *11–14***). This senescence phenotype is much more

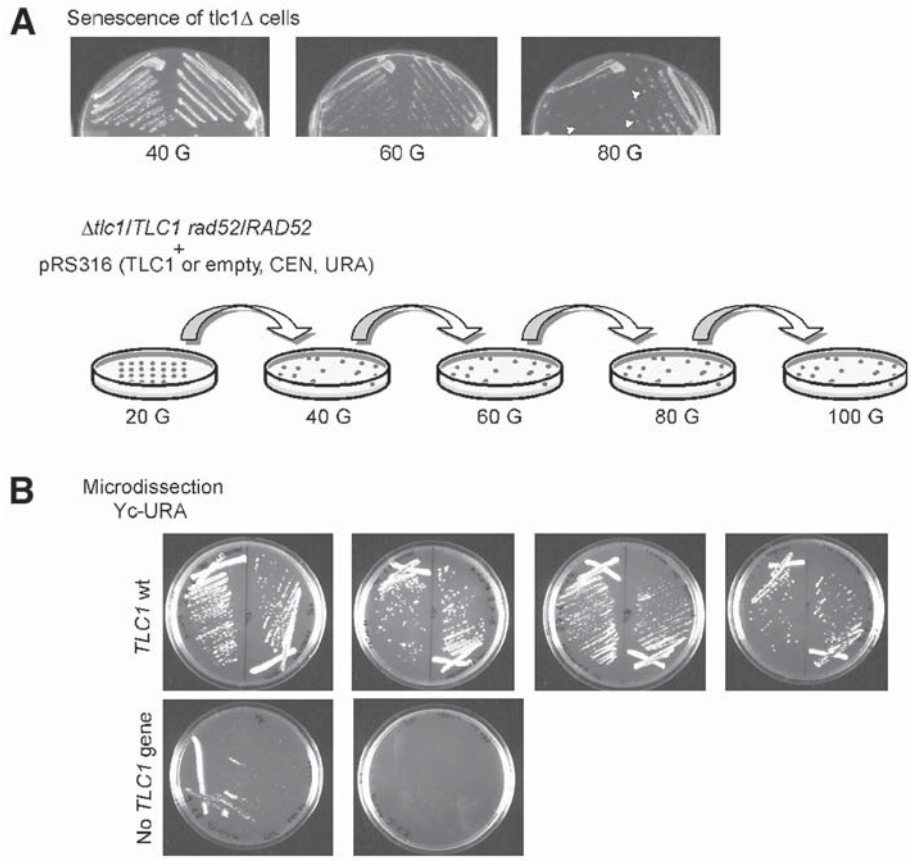

Fig. 2. Plates with passages of a senescent yeast strain to show appearance of survivors and senescence of a *rad52Δtlc1Δ* strain. **(A)** Yeast strain RWY12 (*Mat***a**, *ura3-52*, *lys 2-801*, *ade2-101*, *trp1-Δ63*, *his3-200*, *leu2-Δ1*, *VR-ADE2-Telo*, *tlc1Δ*) initially contained a plasmid with the wt *TLC1* gene on it (pRS316: *CEN/ARS*, *TLC1*, *URA3*). Cells were streaked on FOA plates to select cells that had lost the plasmid and then further streaked on YC-plates to assay senescence. All plates were incubated at 30°C for 3 d. Far left plate, appearance of second restreak (relatively healthy looking colonies/streaks); middle plate, appearance of third restreak (the majority of cells are senescing at this stage and isolated colonies are hard to obtain); far right plate, appearance of fourth restreak (most cells do not form colonies anymore, but isolated healthy looking colonies are visible, these are survivors (indicated by white triangles). **(B)** Top, schematic drawing of the experimental procedure to obtain different passages starting with double heterozygous diploid cells. Bottom, appearance of restreaks on YC-URA plates. The haploid containing both deletions (*rad52Δtlc1Δ*) and the plasmid lacking the *TLC1* gene only grows for 20–30 generations and dies afterward (second restreak, 40G). No survivors are generated in this setup.

robust and occurs more rapidly (after 20–40 generations) when deletions of genes required for telomerase are combined with a *RAD52* deletion. This combination in general will abort the generation of survivors.

Observations of the senescence phenotype can be obtained either on plates or in liquid cultures. The assay on plate will require restreaking different clones on selective media, letting them grow at the required temperature, followed by a thorough examination of colony growth characteristics. It is easy and not labor-intensive, but very prone to variations and not very quantifiable. The assay based on the same principle can be done in liquid cultures, where the growth of the culture is monitored through successive measuring of culture densities (OD_{600}). In order to obtain the required population doublings, the cultures are diluted every 12–48 h and regrowth is monitored. The senescence of the culture is indicated by a marked slow-down of growth for quite an extended period of time (sometimes days), followed by resumption of growth (indicating the culture has been overtaken by survivors). Thus, the liquid assay is preferable for quantitative measurements of the generations to senescence. However, type I survivors (slower-growing) often are rapidly replaced by type II survivors ***(13)***. In parallel with the culture assays for senescence, telomere lengths can be assayed to document the shortening of telomeres and the subsequent appearance of survivors (*see* **Subheadings 2.1.1.–2.1.8.** and **3.1.1.–3.1.8.**).

1.3. Terminal DNA-Structure Analysis

The end of chromosomes consists essentially of a double-strand DNA portion from which the strand with the 3'-end protrudes. We call these extensions G-tails, because they are invariably formed of the G-rich strand of the telomeric repeats (*see* **Subheadings 1.1., 2.1.1.–2.1.8.**, and **3.1.1.–3.1.8.**). This end-structure is a conserved motif at the ends of chromosomes in a variety of organisms. In the yeast *Saccharomyces cerevisiae*, G-tails normally are about 10–15 nt long and become much longer in late S-phase, after conventional replication has been completed ***(15,16)***. The terminal DNA arrangement at telomeres can be visualized by an in-gel hybridization technique originally developed in our laboratory ***(17)***, and despite a generally held misconception, the technique can be easily performed outside our own laboratory and give wonderful results. It can be applied on DNA isolated from non-synchronous yeast cultures, or from DNA derived from S-phase enriched cultures (*see* Chapter 18 on 2-D analyses of replication intermediates).

Native conditions used for the technique allow the detection of genomic single-stranded DNA. Double-stranded DNA is not detected in such conditions, but can be revealed after denaturing DNA in the gel as a control. The in-gel hybridization technique is a powerful tool to characterize yeast mutant strains that have altered DNA end-structures (for example *yku70Δ, yku80Δ,*

cdc13-1, rad27Δ, pol1-17 strains show this phenotype) ***(18–22)***. The technique can also be applied to other organisms, such as mammalian cells, for the detection of G-tails ***(23)***.

The in-gel hybridization technique uses an agarose gel to separate DNA molecules according to their molecular weight, such as TRFs (*see* **Subheadings 1.1., 2.1.1.–2.1.8.**, and **3.1.1.–3.1.8.**). The DNA molecules are then hybridized directly in the gel to an end-labeled oligonucleotide. This procedure requires drying of the agarose gel before hybridization, which is a critical step in the experiment. Single- and double-stranded DNA, as well as genomic DNA treated by nuclease, such as Exonuclease I, must be used in parallel to native DNA in order to validate the particular experiments.

1.4. Telomere PCR and Sequencing

Yeast telomeres undergo shortening and lengthening, two processes that are tightly regulated in order to maintain telomere length at a constant mean. As for many other eukaryotic organisms, it is a domain of the RNA subunit of the telomerase ribonucleoprotein (RNP), which specifies the addition of the appropriate repeat-sequence to telomeres. This domain in the RNA contains a stretch of sequence that is complementary to the telomeric DNA sequence. Thus, modifications within the template domain can lead to changes in the incorporation of repeat DNA ***(24,25)***. In addition, substitution of the entire yeast telomerase RNA template for a human one directs the synthesis of vertebrate telomeric DNA onto yeast chromosomal ends ***(26,27)***.

Such changes in the nature of telomeric repeats as well as a more quantitative estimation of telomere length may require sequencing actual telomeric repeats. For this purpose, a polymerase chain reaction (PCR)-mediated method has been developed ***(28)***. The method takes advantage of the fact that the single-stranded 3'-overhang at chromosome ends is an appropriate substrate for the Terminal deoxynucleotidyl transferase enzyme (TdT), a DNA polymerase that catalyzes the repetitive addition of deoxynucleotides to the 3'-OH termini of DNA. Compared to other protocols used for cloning and sequencing telomeres ***(5,29,30)***, this technique provides a unique method for 3' end-labeling of DNA molecules and allows the rapid analysis of length and sequence of a complete chromosomal telomere. Owing to the nature of the PCR reaction, no distinction between the double-stranded and single-stranded regions of DNA can be made with this assay.

1.5. Telomerase Assay

Maintenance of both telomere length and structure is crucial for cell viability and the telomerase enzyme is a key player for this purpose. The telomerase RNP consists of a catalytic core containing a reverse transcriptase-like protein

(TERT, Est2p in yeast) and an integral RNA component (TR, TLC1 in yeast), which serves as template for telomeric repeat addition onto the G-rich strand. These two are the only subunits required for in vitro telomerase activity. However, the proteins Est1p, Est3p, and Cdc13p are also required to form an active telomerase holoenzyme in vivo ***(24,31–34)***. In order to characterize telomerase activity in particular mutants vs wild-type yeast strains, one can use an in vitro telomerase activity assay. For example, mutations, deletions, or insertions in the *TLC1* RNA and their effect on telomerase activity can be studied using such an assay. Furthermore, mutations or deletions in various components involved in modifying and regulating the telomerase complex can be studied. Finally an increase or decrease in telomerase activity in particular experimental settings can be determined. The methods reported for ProA-*EST2* construction, Western blotting, immunoprecipitation, and telomerase activity assay were modified from ***(35)***.

1.6. Other Assays Related to Telomere Maintenance

1.6.1. Chromatin Immunoprecipitation Assay With or Without Quantitative PCR

This assay is used to detect in vivo protein–DNA interactions. Briefly, the protein studied is crosslinked in vivo to others and to DNA using formaldehyde. The crosslinked DNA is sheared into small pieces by sonication and whole cell extract is immuno-precipitated with a specific antibody against the protein of interest (these days, antibodies against specific protein tags are frequently used). The protein–DNA crosslinks are reversed, the DNA is deproteinized, and the precipitated DNA is analyzed, either directly by Southern blotting or by PCR-amplification and subsequent analysis.

The original paper using this method on telomeres demonstrates that Sir2p, Sir3p, Sir4p, and Rap1p map to the same sites along telomeric heterochromatin in wild-type cells ***(36)***. The assay has also been used to show the association of yeast Ku protein to telomeric DNA ***(19)***, and to show the telomeric binding of Est1p, Est2p, and Cdc13p during the cell cycle ***(37)***. Furthermore, the presence of RPA at yeast telomeres, with a maximal association in S phase, was confirmed ***(38)***, and different yeast proteins associated to a humanized telomere present in *S. cerevisiae* were detected ***(39)***.

1.6.2. Silencing and Telomeric Position Effect

Telomeric position effect (TPE) in wild-type yeast is the phenomenon where transcription of genes next to telomeres is reversibly repressed ***(40)***. The transcriptional state of a telomere-linked gene is reversible, and once established both the transcribed and repressed states are stable for many cell generations

(40,41). TPE is often monitored by determining the fraction of cells with a telomeric marker gene (either *URA3* or *ADE2*) that are able to grow on a determined selective media. For example, media containing 5-fluoroorotic acid (FOA), a compound that kills cells when Ura3p is expressed, can be used as an indicator of repression of a telomeric *URA3* gene. Moreover, the repression of a telomeric *ADE2* gene will lead to the formation of red colonies on YEPD plates containing low concentrations of adenine.

This method is widely used to determine if particular proteins are implicated in the formation and maintenance of telomeric heterochromatin. In early studies, loss of silencing was achieved using mutations in SIR or histone genes ***(42,43)***, thereby affecting all telomeres. More recent studies have adapted this technique to eliminate TPE in cis at a single telomere ***(44)***. It was also shown that silencing is reduced at a humanized telomere contained in yeast *S. cerevisiae* compared to wild-type telomeres ***(39)***, and that TPE is reduced in the polα ts mutant ***(18–22)***. Moreover, there is mounting evidence for a higher order organization of yeast telomeric chromatin, which can play a role in regulating TPE ***(45)***, as well as being the yeast counterpart of the mammalian t-loop. A disadvantage of the technique is the fact that it is not quite clear what exactly is measured when TPE is assayed. Many factors and processes affect chromatin and telomeric loci and TPE may be affected indirectly by them. In addition, there is evidence that transcriptional regulation at native telomeres containing subtelomeric repeats is not subject to a linear and gradually decreasing repression over the region ***(46)***.

1.6.3. One-Hybrid Assays

The one-hybrid assay uses a protein of interest fused to a transcriptional activation domain, and tests for its ability to activate transcription of a promoter-defective specific allele on selective media. Transcriptional activation by the protein of interest would be accomplished by its binding to a region specific chromatin and/or DNA. The locus-specific effect of the protein of interest is compared to effects induced by other proteins or when the indicator gene is moved to other locations in the genome.

The one-hybrid assay for telomere binding proteins was described and used to establish that six proteins that affect telomere structure or function (but that had not been shown previously to bind telomeres in vivo) are indeed telomere binding proteins ***(47)***. A promoter-defective allele of *HIS3* was placed adjacent to a chromosomal telomere. Candidate proteins fused to a transcriptional activation domain were tested for the ability to activate transcription of the telomere-linked *HIS3* gene ***(47)***. More recently, Rfa2p binding was shown to require the presence of a chromosome end in vivo ***(38)***.

1.6.4. Healing Assay

This assay was developed to visualize the addition of telomeric sequence onto a *de novo* telomere created in vivo. The assay relies on the induction of the HO endonuclease placed under the control of a galactose-inducible promoter to create a double-strand DNA break (DSB) on a chromosome. For example, the recognition site was placed immediately adjacent to the 81 base pairs of $TG_{1–3}$ and the cleavage liberated a 4-nucleotide 3' TG tail overhang, similar to what can be present on native telomeres. Healing events, resulting from telomere addition at the site of DSB can then be selected and analyzed ***(48,49)***. This in vivo assay allows more insights into the mechanistic of telomerase functions, as well as the study of telomere addition during the cell cycle and the proteins required for this process ***(49,50)***.

2. Materials

2.1. Telomere Length Assay

2.1.1. Yeast Cultures and DNA Preparation

1. YEP (rich media without any selection) or YC (synthetic media) lacking any amino acid to ensure the selection of a given plasmid or genomic marker gene.
2. 20% D-glucose (BioShop, Burlington, Canada).
3. Culture tubes and 16 ×100 mm glass tubes.
4. Sterilized toothpicks.
5. Roller drum in a room kept at 30°C.
6. Sterile water.
7. Lysis Buffer for glass bead DNA isolation protocol: 100 m*M* Tris-HCl, pH 8.0, 50 m*M* disodium ethylenediamine tetraacetate dihydrate (EDTA), 1% sodium dodecyl sulfate (SDS). Store at room temperature.
8. Acid-washed glass beads: these 0.45–0.5-mm glass beads can be purchased from Sigma (Sigma, Oakville, Canada). The beads are cleaned by soaking in 0.25 *M* Hydrochloric acid (HCl) for 2 h, then washed extensively with water until the pH of the supernatant reaches approx 6.5. The beads are allowed to dry at room temperature on the counter and autoclaved in glass beakers before use.
9. Vortex apparatus.
10. 5 *M* Sodium chloride (NaCl).
11. Phenol:chloroform:isoamyl alcohol (25:24:1) saturated with 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
12. Chloroform:isoamyl alcohol 24:1.
13. Cold 100% ethanol.
14. Ribonuclease A (Amersham Biosciences, Baie d'Urfé, Canada; RNase A; 10 mg/mL): Dissolve RNase A to 10 mg/mL in 10 m*M* sodium acetate (pH 5.0–5.2 adjusted with acetic acid). Boil for 15 min and slowly let cool to room tempera-

ture. Adjust the pH by adding 0.1 vol of 1 *M* Tris-HCl, pH 7.4. Store in 500-µL aliquots at –20°C.
15. Proteinase K (Bioshop; 10 mg/mL): dissolve in water to 10 mg/mL and store in 100-µL aliquots at –20ºC.
16. TNE solution: 10 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 1 m*M* EDTA. Store at room temperature.
17. Cold 70% ethanol.
18. TE, pH 8.0: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
19. NIB solution (Nuclear Isolation Buffer): 17% (w/v) glycerol (Sigma), 50 m*M* (3-[N-Morpholino]propanesulfonic acid) sodium salt (MOPS), pH 7.5, 150 m*M* CH_3CO_2K, 2 m*M* $MgCl_2$, 500 µ*M* spermidine (Sigma), 150 µ*M* spermine (Sigma). Autoclave and store at 4ºC.
20. Lysis Buffer (for NIB protocol); 50 m*M* Tris-HCl, pH 8.0, 20 m*M* EDTA, 100 m*M* NaCl.
21. 10% Sarkosyl; 10 g of *N*-lauroyl-sarcosine (Sigma) in 100 mL of H_2O. Sterilize by filtration and store at room temperature.

2.1.2. DNA Dosage, Digestion, and Controls

1. Hoechst dye solution (1 mg/mL): dissolve 10 mg of Hoechst 33258 (Sigma) in 10 mL sterile water. Do not filter. Store at 4ºC for up to 6 mo in a dark bottle (*see* **Note 1**).
2. 10X TNE buffer: 100 m*M* Tris-HCl, 10 m*M* EDTA, 2 *M* NaCl. Adjust the pH to 7.4. Filter before use and store at 4ºC for up to 3 mo (*see* **Note 2**).
3. Fluorometer apparatus and appropriate cuvettes.
4. DNA standards: use a DNA standard stock solution at 500 ng/µL (such as the 1 kb ladder from Gibco, Burlington, Canada).
5. Parafilm.
6. Low-lint tissue.
7. Water.
8. 10X "magic" buffer: 200 m*M* Tris-HCl, pH 7.2, 700 m*M* NaCl, 200 m*M* KCl, 100 m*M* $MgCl_2$, 0.5 m*M* spermine, 0.125 m*M* spermidine, 0.1% aprotinin (Sigma). Sterilize by filtration and store at 4°C.
9. 1% digitonin: dissolve 1g of digitonin (Sigma) in 100 mL H_2O. Sterilize by filtration and store at 4°C.
10. 0.1 *M* DTT (D-L-Dithiothreitol from Sigma): solubilize in water, filter-sterilize, and store in 500-µL aliquots at –20°C.
11. RNase A (5 mg/mL): dissolve RNase A to 5 mg/mL in sodium acetate 10 m*M* (pH 5.2 adjusted with acetic acid). Boil for 15 min and let cool slowly to room temperature. Adjust the pH by adding 0.1 volume of 1 *M* Tris-HCl, pH 7.4. Store in 500-µL aliquots at –20°C.
12. *Xho*I enzyme (New England Biolabs, Mississauga, On., Canada).
13. Stop buffer: 20 m*M* EDTA, pH 8.0, 0.3 *M* NaCl.
14. Phenol:chloroform.

15. Cold 100% ethanol.
16. Cold 70% ethanol.
17. 10X DNA loading buffer: 50% (w/v) glycerol (Sigma), 0.25% (w/v) bromophenol blue (Sigma), 0.25% (w/v) xylene cyanol FF (Sigma), 0.1 *M* EDTA, pH 8.0. Store at 4ºC.

2.1.2.1. Bal 31 Treatment

1. 5X Bal 31 buffer: 100 m*M* Tris-HCl, pH 8.0, 60 m*M* $CaCl_2$, 60 m*M* $MgCl_2$, 5 m*M* EDTA, pH 8.0, 3 *M* NaCl.
2. Bal 31 enzyme (Promega, Madison, WI).
3. 0.25 *M* Ethylene glycol-*bis*(β-aminoethylether)-*N*,*N*,*N*',*N*'-tetraacetic acid tetrasodium salt (EGTA; Sigma).
4. Block heater or water bath at 65ºC.
5. Phenol:chloroform.
6. Cold 100% ethanol.
7. TE, pH 8.0.

2.1.3. Gel Electrophoresis

1. Agarose LE (Roche Diagnostics Corporation, Indianapolis, IN).
2. Masking tape.
3. Microwave oven.
4. Electrophoresis buffer:
   a. 50X TAE (Tris, acetate, EDTA): 2 *M* Tris-base, 1 *M* glacial acetic acid, 50 m*M* EDTA, pH 8.0; the solution should be at approx pH 7.9.
   b. 5X TBE (Tris, borate, EDTA): 450 m*M* Tris-base, 450 m*M* boric acid (Fisher Scientific, Nepean, On., Canada), 10 m*M* EDTA, pH 8.0. The pH of the solution should be approx 8.4.
5. Ethidium bromide (EtBr from Roche Diagnostics Canada, Laval, Qc., Canada; 10 mg/mL): The stock solution is obtained by dissolving 1g of EtBr in 100 mL H_2O. Stir on a magnetic stirrer for several hours to allow complete dissolution (*see* **Note 3**). Wrap the bottle in aluminum foil and store at room temperature. Dilute the stock solution 1:20,000 for gels or staining solutions.
6. 10X loading buffer: 50% (w/v) glycerol (Sigma), 0.25% (w/v) bromophenol blue (Sigma), 0.25% (w/v) xylene cyanol FF (Sigma), 0.1 *M* EDTA, pH 8.0. Store at 4ºC.
7. DNA molecular weight markers:
   a. 1 kb DNA ladder (Gibco).
   b. Lambda DNA/*Hin*dIII digested (Amersham Biosciences).
8. Electrophoresis apparatus and power supply.
9. Gel doc apparatus (Bio-Rad, Mississauga, On., Canada).

2.1.4. Transfer of the DNA Onto a Nylon Membrane

1. Scalpel blade.
2. Glass container (large enough to contain the gel).

3. Depurination solution: 0.25 *N* HCl in H_2O (*see* **Note 4**). Store at room temperature.
4. Denaturation solution: 1.5 *M* NaCl, 0.5 *M* NaOH. Store at room temperature.
5. Transfer solution: 0.4 *M* NaOH.
6. Nylon membrane, Hybond-N+ (Amersham Biosciences).
7. 20X SSC (sodium chloride/sodium citrate): 3 *M* NaCl, 300 m*M* sodium citrate–$2H_2O$. Dissolve in H_2O and adjust the pH to 7.0 by adding approx 75 mL of concentrated HCl. Autoclave and store at room temperature.
8. 100X Denhardt's solution: 2% (w/v) polyvinylpyrrolidone (PVP; Sigma), 2% (w/v) Ficoll (Amersham Biosciences), 2% (w/v) bovine serum albumin (BSA). Dissolve in H_2O. Sterilize by filtration and store in 50-mL aliquots at –20ºC.
9. Formamide (Fisher Scientific).
10. Herring sperm DNA, 50 mg/mL, degraded free-acid (Sigma). Dissolve in H_2O to 50 mg/mL. Just before use, the solution is heated for 5 min in a boiling water bath and then quickly chilled on ice.
11. Whatman paper (five pieces approximately the size of the gel).
12. Transfer apparatus (*see* **Note 5**).
13. Sealer and heat-sealable bags.

2.1.5. Labeling and Purification of DNA Probes

2.1.5.1. Random Prime Labeling of DNA Fragments

1. DNA fragment to be used; in solution at 100 ng/µL.
2. TM solution: 250 m*M* Tris-HCl, pH 8.0, 25 m*M* β-Mercaptoethanol (Sigma), 25 m*M* $MgCl_2$.
3. Primer stock ($pd(N)_6$, sodium salt; Amersham Biosciences): dissolve random hexanucleotides in 500 µL TE, pH 8.0. Store at –20ºC.
4. 1 *M* HEPES, pH 6.6.
5. LS-buffer: 25 µL TM solution, 25 µL 1 *M* HEPES, pH 6.6, 7 µL primer stock ($pd(N)_6$). Keep at –20ºC.
6. SDS-EDTA solution: 0.2% SDS, 50 m*M* EDTA.
7. Block heater at 100ºC or boiling water.
8. dATP (Amersham Biosciences), dGTP (Amersham Biosciences), dTTP (Amersham Biosciences) mix: 1 m*M* dATP, 1 m*M* dGTP, 1 m*M* dTTP in water and store at –20ºC.
9. Klenow enzyme (Amersham Biosciences).
10. α^{32}P-dCTP (PerkinElmer Life Sciences; Woodbridge, On., Canada) (250 µCi [9.25 MBq]; 800 Ci/mmol).
11. Block heater or water bath at 65ºC.

2.1.5.2. 5'-Oligo Labeling

1. Oligo to be used in solution at 100 ng/µL.
2. 10X T4 polynucleotide kinase (PNK) buffer.
3. γ^{32}P-ATP (PerkinElmer Life Sciences; 250 µCi [9.25 MBq]; 3000 Ci/mmol).
4. T4 polynucleotide kinase (Amersham Biosciences).

5. Block heater or water bath at 65ºC.
6. TE, pH 8.0.

2.1.5.3. Purification of Probes

1. Spin column of G-50 Sephadex (Amersham Biosciences).
2. Microcentrifuge.

2.1.6. Prehybridization and Hybridization

2.1.6.1. Random Probe

1. Superstock (hybridization solution): 50% formamide (Fisher), 5X SSC, 1X Denhardt's solution, 1 mg/mL herring sperm DNA, 2% SDS, 0.5% low-fat milk powder (Carnation) in sterile H_2O. Store in 50-mL aliquots at 4ºC.
2. Dextran sulfate.
3. Radiolabeled probe.

2.1.6.2. 5'-End Labeling of Oligonucleotides

1. 5' labeled oligonucleotide.
2. In-gel hybrydization solution: *see* composition in **Subheading 2.3.7.**
3. Sealer and heat-sealable bags.
4. Water bath at 37ºC.

2.1.7. Washing of the Membrane and Exposition

2.1.7.1. Washing Conditions for Random Probe

1. 2X SSC.
2. Plastic container (tupperware) large enough for the membrane.
3. Shaking platform at room temperature.
4. 0.1X SSC + 0.1% SDS.
5. Water bath at 55ºC.
6. 0.1X SSC.
7. Autoradiography cassettes and amplifying screens.
8. Autoradiogram (films).
9. –80ºC freezer.

2.1.7.2. Washing Conditions for 5'-End-Labeled Oligo-Probe

1. Shaking platform at room temperature.
2. 0.25X SSC.
3. Sealer and heat-sealable bags.
4. Autoradiography cassette and amplifying screens.
5. Autoradiogram (films).
6. –80ºC freezer.

2.1.8. Stripping of the Membrane

1. Transfer solution: 0.4 *M* NaOH.

2. Stripping solution: 0.1X SSC, 0.1% SDS, 0.2 *M* Tris-HCl, pH 7.5.
3. Water bath at 45ºC.
4. Plastic container.

2.2. Yeast Senescence Assays

2.2.1. Yeast Streaking on Selective Media and Growth of Different Yeast Strains

1. Sterilized toothpicks.
2. Sterile plates with the required medium.
3. Incubator set at the required temperature (23ºC, 30ºC, 37ºC).

2.2.2. Colony Analysis on Plates

1. Plates with successive passages of the strain(s) to study.

2.2.3. Appearance of Survivors and Analysis of Their Phenotypes

1. Plates with the successive passages of the strain(s) to study.
2. Photographic equipment suitable for yeast plates.

2.2.4. Survivor Analysis in Liquid Cultures

1. Sterile glass tubes.
2. Sterile toothpicks.
3. Sterile liquid media.
4. Bunsen burner.
5. Rotating platform at the required temperature.
6. Spectrophotometer and cuvets.

2.3. Terminal DNA–Structure Analysis

2.3.1. Yeast Cultures and DNA Preparation (see **Subheading 2.1.1.**)

2.3.2. Restriction Enzyme Digestion of DNA (see **Subheading 2.1.2.**)

2.3.3. DNA Controls

2.3.3.1. Single-Stranded DNA Controls

Single-stranded DNAs used as controls for native gels are derived from pCA75 and pGT75 plasmids ***(15)***. Both plasmids contain 72 bp of $C_{1-3}A/TG_{1-3}$ DNA (originally cloned from a natural yeast telomere) inserted into the *Bam*HI site of pVZ1 ***(51)*** in opposite orientations (*see* **Note 6**).

2.3.3.2. Double-Stranded DNA Control

Control for double-stranded DNA is derived from plasmid pRS303 ***(52)***, into which 55 bp of $C_{1-3}A/TG_{1-3}$ DNA repeats were inserted into the *Eco*RV site, resulting in a plasmid called pMW55 ***(17)***.

2.3.3.3. Genomic DNA Treatments

2.3.3.3.1. Exonuclease I Treatment

1. *Escherichia coli* Exonuclease I (USB; 10 U/µL). Store at –20°C.
2. 10X ExoI buffer: 100 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, pH 8.0, 100 m*M* $MgCl_2$, 200 m*M* KCl, 100 m*M* β-mercaptoethanol. Store at –20°C.
3. 2X Stop buffer: 30 m*M* EDTA, pH 8.0, 200 m*M* NaCl.
4. Phenol/chloroform: 1 vol of phenol, pH 8.0, mixed with 1 vol of chloroform. Store at 4°C.
5. TE: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0.

2.3.3.3.2. Mung Bean Nuclease Treatment

1. Mung bean nuclease (Amersham Biosciences; 147.2 U/µL). Store at –20°C.
2. 10X Mung bean buffer: 300 m*M* sodium acetate, pH 4.5, 500 m*M* NaCl, 10 m*M* $ZnCl_2$, 50% glycerol. Store at –20°C.
3. 2X Stop buffer: 30 m*M* EDTA, pH 8.0, 200 m*M* NaCl.

2.3.4. Labeling of DNA Probes and Purification

2.3.4.1. 5'-End Labeling of Oligonucleotides (*see* **Subheading 2.1.5.2.**)

2.3.4.2. Labeling by Random Priming (*see* **Subheading 2.1.5.1.**)

2.3.4.3. Purification of Probes (*see* **Subheading 2.1.5.3.**)

*2.3.5. Gel Electrophoresis (*see ***Subheading 2.1.3.****)*

2.3.6. Gel Drying

1. Bio-Rad 583 gel dryer.
2. 2X SSC: 300 m*M* NaCl, 30 m*M* sodium citrate, pH 7.0.
3. 3MM Wathman paper.
4. Plastic wrap.
5. Dry ice and 100% ethanol.

2.3.7. In-Gel Hybridization Procedure

1. In-gel Hybridization solution: 5X SSC, 5X Denhardt's solution, 0.1X P-wash, 0.04 µ*M* ATP, 20 µg/mL denatured salmon sperm DNA (ssDNA). Store at 4°C.
2. 5X SSC: 750 m*M* NaCl, 75 m*M* sodium citrate, pH 7.0.
3. 5X Denhardt's solution: 0.2% (w/v) Ficoll, 0.2% (w/v) PVP, 0.2% (w/v) BSA. Store at –20°C.
4. 0.1X P-wash: 0.5 m*M* pyrophosphate, 10 m*M* Na_2HPO_4. Store at 4°C.
5. Oligonucleotide probes: CA-probe 5'-CCCACCACACACACCCACACCC-3' is used to detect the presence of G-tails. GT-probe 5'-GGGTGTGGGTGTGTG TGGTGGG-3' is used as control.
6. Sealer and heat-sealable bags.
7. Water bath at 37ºC.

2.3.8. Washing Conditions

2.3.8.1. Gel Washing After Hybridization

1. 0.25X SSC: 37.5 m*M* NaCl, 3.75 m*M* sodium citrate, pH 7.0.
2. Plastic container.
3. Shaking platform at room temperature or other temperature.

2.3.8.2. Probe Removal in Native Conditions

1. 0.25X SSC: 37.5 m*M* NaCl, 3.75 m*M* sodium citrate, pH 7.0.
2. Plastic container.
3. Water bath at 37°C or higher temperature.

2.3.9. Denaturation of DNA and Rehybridization

2.3.9.1. Denaturation of DNA and In-Gel Rehybridization

1. Denaturing solution: 150 m*M* NaCl, 0.5 *M* NaOH.
2. Neutralizing solution: 150 m*M* NaCl, 0.5 *M* Tris-HCl, pH 8.0.

2.3.9.2. DNA Transfer by Southern Blotting and Probing (*see* **Subheadings 2.1.4.**, **2.1.6.**, and **2.1.7.**)

2.4. Telomere PCR and Sequencing

*2.4.1. Yeast Strains, Culture, and DNA Ppreparation (*see ***Notes 7*** *and* ***8****, and Refer to* ***Subheading 2.1.1.****)*

2.4.2. Tailing of the 3' End

1. Terminal deoxynucleotidyl Transferase (TdT), FPLC*pure* from Amersham Biosciences. Dilute TdT enzyme to a final concentration of 20 U/µL in 1X TdT buffer from Gibco. Keep at –20ºC.
2. 5X tailing reaction buffer from Gibco (500 m*M* potassium cacodylate, pH 7.2, 10 m*M* $CoCl_2$, 1 m*M* DTT), stored at –20ºC (*see* **Note 9**).
3. 2'-deoxycytidine-5'-triphosphate (dCTP) from Amersham Biosciences, supplied as a 100 m*M* solution in water, pH 7.5. Dilute dCTP to 10 m*M* in water.
4. Tailing mix: 1 U TdT per reaction, 2 µL of 5X TdT buffer, 1 µL of 10 m*M* dCTP, and X µL H_2O (*see* **Note 10**).

2.4.3. PCR Amplification of 3' End-Tailed Telomeres

1. Thermal cycler.
2. Primers (we buy them from Gibco, *see* **Note 11**) are diluted to 20 pmol/µL. **DIA5-1: 5'-**GTGAGCGGATAACAATTTCACACAG*TCTAGA*TGTCCGAATTGATCCCAGAGTAG-3' and **dG_{18}-*Bam*HI**: 5'-CG*GGATCC*$(G)_{18}$-3'.
3. puReTaq™ Ready-To-Go™ PCR Beads (Amersham Biosciences; *see* **Notes 12** and **13**).

2.4.4. Analysis of PCR Products

1. Gel electrophoresis apparatus and power supply (refer to **Subheading 2.1.3.**).
2. DNA gel extraction kit (Qiagen).

2.4.5. Cloning

1. pGEM-T vector system I from Promega.
2. DH5α competent cells
3. 1 *M* glucose: dissolve 18 g of glucose in 90 mL of deionized water. After glucose has dissolved, adjust the volume to 100 mL with water and sterilize by filtration through a 0.22-micron filter and store at room temperature.
4. SOC medium: 20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl in 1 L of water. Autoclave and let cool to 60ºC. Subsequently add 20 mL of sterile 1 *M* glucose.
5. Luria-Bertani (LB) plates with ampicillin, spread over with 100 µL of 100 m*M* Isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma) and 20 µL of 50 mg/mL 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; Sigma).
6. QIAprep Spin Miniprep Kit Protocol form Qiagen.

2.4.6. DNA Sequencing

1. Sequitherm Excel II DNA sequencing kit (Epicentre Technologies). This kit is optimized for sequencing difficult-to-sequence regions, like the GT repeats of yeast telomeres, with several automated sequencers like LI-COR, NEN, ALF, and ABI PRISM.
2. pUC/M13 Forward sequencing primer; pGEM-T vector has a binding site for the pUC/M13 forward primer. Reverse primers can be used also.
3. LI-COR DNA sequencers; we recommend using automated sequencers from this series (LI-COR 4000 and 4200 series).

2.5. Telomerase Assay

2.5.1. Preparation of Yeast Cell Cultures and Protein Extraction

1. Growth media: YEPD is used for strains that do not have any specific markers.
2. TMG buffer for cell lysis: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* $MgCl_2$, 10% glycerol. Once filtered or autoclaved can be stored at 4°C.
3. TMG/NaCl: 5 *M* NaCl is added to TMG buffer to a final concentration of 200 m*M*.
4. DTT (Sigma) is dissolved in H_2O to a working concentration of 0.1 *M* and stored in single-use aliquots at –20°C.
5. TMG/NaCl/DTT: 0.1 *M* DTT must be added to TMG/NaCl buffer to a final concentration of 0.1 m*M*.
6. Protease inhibitors: Complete, Mini, EDTA-free protease inhibitor cocktail tablets (Roche Applied Science). Use the inhibitors as one tablet for every 10 mL of TMG buffer. Dissolve the tablet just before the protein extraction is performed.
7. Acid-washed glass beads, 425–600 µm (Sigma; *see* **Subheadings 2.1.1.** and **2.1.8.**).

2.5.2. Immunoprecipitation of Protein-A Tagged Est2 Protein

1. RNasin; RNase inhibitor from Promega (2500 U; 40 U/µL).
2. Tween-20 from Bio-Rad.
3. IgG sepharose beads (Amersham Biosciences).
4. TMG Buffer A: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* $MgCl_2$, 10% glycerol, 0.1 m*M* DTT. Keep at 4°C.
5. TMG Buffer B: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* $MgCl_2$, 10% glycerol, 0.1 m*M* DTT, 200 m*M* NaCl, 0.5% Tween-20. Keep at 4°C.
6. TMG Buffer C: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* $MgCl_2$, 10% glycerol, 0.5 m*M* DTT. Keep at 4°C.
7. Protease inhibitors: use as described in protein extraction, **Subheading 2.5.1.**
8. 360° Lab Quake rotator at 4°C.

2.5.3. SDS-Polyacrylamide Gel Electrophoresis

1. Prepare stock solutions of 1 *M* Tris-HCl, pH 6.8, and 1.5 *M* Tris-HCl, pH 8.8, in water. Autoclave. Store at room temperature.
2. Thirty percent acrylamide/bis solution (29:1). Store in light-protecting bottles at room temperature (*see* **Note 14**).
3. Ammonium persulfate (APS): prepare a 10% stock solution in water and store at 4°C for 1–2 wk.
4. *N*,*N*,*N*',*N*'-Tetramethyl-ethylenediamine (TEMED, Bio-Rad).
5. Water-saturated isobutanol: Shake equal volumes of water and isobutanol in a glass bottle and let stand to allow separation. Use the top layer. Store at room temperature.
6. 10X Running buffer: 0.25 *M* Tris, 1.92 *M* glycine, 1% (w/v) SDS, in water. Store at room temperature.
7. 2X Laemmli loading buffer: 20% glycerol, 4.6% SDS, 0.125 *M* Tris-HCl, pH 6.8, 0.2% (w/v) bromophenol blue. Store at room temperature. Just before use, add 10% 2-mercaptoethanol under a fume hood.
8. Prestained molecular-weight markers: Kaleidoscope markers (Bio-Rad).
9. Hoefer Mighty Small II electrophoresis apparatus (for mini-gels).

2.5.4. Western Blotting for Detection of proA-Est2p

1. Transfer buffer: 25 m*M* tris, 0.192 *M* glycine, 25% (v/v) methanol. Store at 4°C.
2. Hybond-C nitrocellulose membrane (Amersham Biosciences) and 3MM chromatography paper from Whatman.
3. Phosphate-buffered saline (PBS; 10X stock solution): 1.4 *M* NaCl, 27 m*M* KCl, 100 m*M* Na_2HPO_4, 18 m*M* KH_2PO_4. Adjust to pH 7.3. Stock solution is kept at room temperature. Dilute to 1X with water before use and, at various steps during the Western blot, Tween-20 is added to a final concentration of 0.05% (PBS-T). Keep the 1X PBS or PBS-T at 4°C.
4. Blocking solution: 5% (w/v) nonfat dry milk in PBS-T.
5. Antibody dilution solution: 1% (w/v) nonfat dry milk in PBS.

6. Primary antibody: polyclonal anti-protein A from rabbit (Sigma, cat. no. P3775).
7. Secondary antibody: Anti-rabbit Ig conjugated with horseradish peroxidase, from donkey (Amersham Biosciences).
8. Enhanced chemiluminescent (ECL) reagents from Amersham Biosciences and Bio-Max Blue XB-1 film from Kodak (Rochester, NY).

2.5.5. In Vitro Telomerase Activity Assay

1. Ribonuclease A (RNase A; Amersham Biosciences): 10 mg/mL stock solution. Keep at –20°C for long-term storage. An aliquot can be kept at 4°C for day-to-day use.
2. 30°C water bath or incubator.
3. Master Mix: 290 m*M* Tris-HCl, pH 8.0, 360 m*M* NaCl, 35% (v/v) glycerol, 18 m*M* $MgCl_2$, 3.6 m*M* spermidine, 3.6 m*M* DTT. Store at –20°C in aliquots.
4. Stop buffer: 250 m*M* Tris-HCl, pH 8.0, 250 m*M* EDTA, pH 8.0, 2% SDS. Must be made fresh each time.
5. Proteinase K (BioShop): 20 mg/mL stock solution in water. Keep at –20°C.
6. Sequencing Gel 20% Ready Mix (50 mL = 1 gel): dissolve 24 g urea in 5 mL 10X TBE, 25 mL of 40% acrylamide/*bis* solution and complete to 50 mL with ddH_2O. Let dissolve on shaker at room temperature (*see* **Note 15**).
7. Telomeric primer oligonucleotide: 5'-TAG GGT AGT AGT AGG G-3' ordered from Sigma (*see* **Note 16** and **ref. *53***).
8. 12 nucleotide-oligo. Any sequence is fine (*see* **Note 17**).
9. Nucleotide Mix: dTTP, dCTP, dATP at 1 m*M* each. Mix in TE or water (*see* **Note 18**).
10. 5 *M* ammomium acetate stock solution. Store at room temperature.
11. 20 mg/mL glycogen stock solution. Store in aliquots at –20°C. Thaw just before use.
12. Cold 100% and 70% ethanol.
13. Formamide loading buffer: 80% (v/v) formamide, 10 m*M* EDTA, pH 8.0, 1 mg/mL xylene cyanol FF, 1 mg/mL bromophenol blue. Store at room temperature.
14. Phenol/chloroform: mix equal amounts of phenol and chloroform. Equilibrate by extracting several times with 0.1 *M* Tris-HCl, pH 7.6. Store the equilibrated mixture under an equal volume of 0.01 *M* Tris-HCl, pH 7.6, at 4°C in a dark glass bottle (*see* **Note 19**).
15. (α-^{32}P) dGTP: 800 Ci/mmol, 10 mCi/mL (Amersham Biosciences). Try to use the radioactivity when it is very fresh as it decreases exposure time (*see* **Note 20**).
16. (γ-^{32}P) ATP: 3000 Ci/mmol, 10 mCi/mL (NEN Life Sciences, Boston, MA).
17. Sigmacote (Sigma).
18. Sequencing gel apparatus (glasses of 40 cm long, *see* **ref. *54*** for a complete description on how to build up the setting).
19. 65°C and 100°C block heaters.

3. Methods

3.1. Telomere Length Assay

3.1.1. Yeast Cultures and DNA Preparation

3.1.1.1. Yeast Cultures (also *see* **Subheading 2.1.1.**)

1. Using a sterile pipet and working in a sterile environment, aseptically transfer 5 mL of the desired media to glass tubes (*see* **Note 21**).
2. With the round end of a sterile toothpick, gently touch a single colony on your plate and transfer it into the tube containing the media by gently rubbing the toothpick against the side of the tube where the media is (*see* **Note 22**).
3. Place the tube on a roller drum at 60 rpm, 30ºC. Let grow for overnight or until the culture is freshly saturated (density of 1–2 × 10^9 cells/mL, measured at 660 nm using a spectrophotometer (corresponds to OD_{660} ~0.6–1.0).

Multiple techniques are available for DNA extraction; the two of them that we use most and that give excellent results will be described in detail.

3.1.1.2. Glass Bead Preparation of Yeast Genomic DNA

1. Spin cell cultures 2 min at 1600*g* using a Sorvall T 6000D, H1000B rotor (*see* **Note 23**).
2. Wash the cell pellet by a 1600*g* using a Sorvall T 6000D, H1000B rotor.
4. Resuspend the cell pellet in 500 µL of lysis buffer. Transfer the solution to 16 × 100-mm glass tubes (*see* **Note 24**).
5. Put tubes on ice (all manipulations from here to **step 8** are on ice; *see* **Note 25**). Add acid-washed glass beads to about 2 mm below the meniscus.
6. Vortex the tubes for 30 s at maximal speed, put on ice, add 25 µL of 5 *M* NaCl to each tube and vortex the tubes twice for 30 s, with a 30-s break on ice between each vortexing step.
7. Remove cells with a 1000-µL pipet tip and transfer to a microcentrifuge tube (*see* **Note 26**).
8. Wash glass beads by adding 200 µL lysis buffer and vortex 5 s; pool the solution with the 500 µL recovered from **step 7**.
9. Add 450 µL of phenol-chloroform, vortex 10 s; spin 5 min in a microcentrifuge. Tranfer the aqueous phase to a new Eppendorf tube and repeat the phenol-chloroform extraction (*see* **Note 19**).
10. Add 1 mL of 100% cold ethanol to supernatant. Vortex; chill at –80°C for 20 min.
11. Spin 15 min in a microcentrifuge, discard the supernatant, and let the pellet dry.
12. Resuspend the pellet in 50 µL TE, pH 8.0, add 2.5 µL RNase A (10 mg/mL), incubate at 37°C for 30 min.

13. Add 150 µL TNE and 5 µL proteinase K (10 mg/mL); incubate at 37°C for 1 h.
14. Extract twice with 200 µL phenol-chloroform (*see* **step 9**).
15. Extract once with 200 µL chloroform (as in **step 9**).
16. Add 2 vol of 100% cold ethanol to supernatant. Vortex; chill at –80°C for 20 min.
17. Spin 15 min in a microcentrifuge, discard the supernatant. Add 1 mL of 70% cold ethanol; spin 5 min, discard supernatant, and let the pellet dry.
18. Resuspend the pellet in 50 µL TE, pH 8.0.
19. Use 1 µL to estimate the concentration of DNA on an agarose gel or to dose directly using the fluorometer (*see* **Subheading 3.1.2.**).

3.1.1.3. NIB-Extraction of Yeast DNA (*see* **Note 27**)

1. Harvest cells in mid-log phase (OD_{660} ~0.6–1.0) by spinning at 1600*g* using a Sorvall T6000D, H1000B rotor for 2 min (*see* **Note 23**).
2. Resuspend cells in ice-cold NIB solution such that there are $1.5–2.0 \times 10^9$ cells/mL. Keep tubes on ice from now on.
3. Add about an equal volume of glass beads, such that the glass beads are within 2 mm of the meniscus (*see* **Notes 24** and **25**).
4. Vortex at setting 8 for 30 s, place the tubes on ice for 30 s. Repeat the procedure 5–20 times.
5. Transfer the liquid into an Eppendorf tube using a P1000 pipet.
6. Wash the glass beads three times with 1 volume of cold NIB solution, combining the washes with the original liquid.
7. Centrifuge at 6000*g* for 10 min at 4ºC in a microcentrifuge.
8. Resuspend the pellet in lysis buffer (same volume as in **step 2**).
9. Add sarkosyl 10% to a final concentration of 1.5% and 2.5 µL of RNase A (10 mg/mL); incubate at 37ºC for 30 min.
10. Add proteinase K (10 mg/mL) to a final concentration of 100 µg/mL and incubate at 37ºC for 1 h.
11. Centrifuge at 9500*g* in a microcentrifuge and keep the supernatant.
12. Add 1 vol of equilibrated phenol to DNA, vortex 10 s, spin 5 min. Keep the aqueous phase.
13. Add 1 vol of phenol-chloroform to DNA, vortex 10 s, spin 5 min. Keep the aqueous phase.
14. Add 1 vol of chloroform to DNA, vortex 10 s, spin 5 min.
15. Precipitate DNA by adding 2 vol of 100% cold ethanol, vortex, and put at –20ºC for 30 min.
16. Spin down DNA for 15 min, wash the pellet with 70% cold ethanol, spin down DNA, and let the pellet air-dry.
17. Resuspend the pellet in 50 µL of TE, pH 8.0.

3.1.2. DNA Dosage, Digestion, and Controls

3.1.2.1. DNA Dosage Using a Fluorometer

1. Start the fluorometer 15 min before use to allow the lamp to stabilize before taking measurements.

2. Wash the cuvet with water and dry the sides with a low-lint tissue. Use 2 mL of the blank solution to standardize the 0-value (*see* **Note 28**).
3. Calibrate the fluorometer using the DNA standard solution (500 ng/µL) in 2 mL of assay solution in the cuvette. Set the factor value to the appropriate setting on your fluorometer and remove the cuvet.
4. Empty and rinse the cuvet. Dry by draining the cuvet and blotting upside down on a paper towel. Add 2 mL of the same assay solution used in **step 2**, insert the cuvet into the well, close the lid, and set 0-value again. After the value is displayed, remove the cuvet.
5. Add 2 µL of sample (*see* **Note 29**) and mix well by inverting the cuvet several times. Place the cuvet into the fluorometer and record the measurement. The units of the obtained value are ng/mL.
6. Repeat **steps 4** and **5** for each sample.

3.1.2.2. Yeast Genomic DNA Digestion to Release TRFs

1. Use between 250 ng and 3 µg of dosed genomic DNA for digestion in a volume of 100 µL and use either the components of the magic buffer or the buffer provided by your enzyme supplier. After some DNA isolation procedures, the magic buffer performs better than the supplied buffers.
2. Mix gently;

X µL genomic DNA	or	X µL genomic DNA
10 µL 10X magic buffer		10 µL recommended 10X buffer
10 µL 1% digitonin		2 µL 5 mg/mL RNase A
10 µL 0.1 *M* DTT		2 µL *Xho*I (40 U)
2 µL 5 mg/mL RNase A		H_2O to 100 µL
2 µL *Xho*I (40 U)		
H_2O to 100 µL		

   Incubate at 37ºC for a minimum of 5 h to overnight.
3. Stop the reaction by adding 1 vol of stop buffer.
4. Extract with phenol:chloroform.
5. Add 2 vol of 100% cold ethanol and place at –20ºC for 30 min.
6. Centrifuge at maximum speed for 15 min. Discard supernatant and wash with 1 vol of 70% cold ethanol.
7. Spin DNA for 10 min and gently discard supernatant.
8. Dry the pellet, resuspend in 1X DNA-loading buffer and load on agarose gel.

3.1.2.3. Bal 31 Treatment

1. Prepare reaction mix (*see* **Note 30**): 2 µg of purified DNA; 20 µL 5X Bal 31 buffer; 1 U Bal 31; H_2O to 100 µL.
2. Incubate the mix at 30ºC and take aliquots at different time points (0, 20, 40, 60, 90, 120 s; 5, 10 min). Stop the reaction by adding 12.5 µL of sample to 2 mL of 0.25 *M* EGTA and incubate at 65ºC for 10 min.
3. Add 90 µL TE and proceed to a phenol:chloroform extraction.
4. Precipitate DNA with 2 vol of 100% cold ethanol, incubate at –20ºC for 30 min and spin 15 min.

5. Discard supernatant and resuspend in 10 µL TE. Proceed to *Xho*I digestion as described in **Subheading 3.1.2.2.**

3.1.3. Gel Electrophoresis

1. Seal the gel-casting platform at both ends with masking tape and insert the comb at the appropriate end.
2. In a bottle that holds at least twice the volume of the final agarose solution, prepare the desired amount of gel to fill the casting platform. Prepare the percentage of gel that is optimal for separation of the desired fragments in either TBE or TAE buffer. For standard *Xho*I-digested DNA and TRF analysis, 0.6% to 1.0% gels work best.
3. Weigh the desired amount of electrophoresis-grade agarose, and complete with the appropriate volume of room temperature buffer. Swirl gently to suspend the agarose.
4. Cover the bottle but do not close it completely. Melt the agarose by boiling several minutes in a microwave (*see* **Note 31**).
5. Swirl the bottle gently to resuspend any agarose particles and reheat until the agarose is completely dissolved. If desired, add ethidium bromide (EtBr) to the gel and electrophoresis buffer at a final concentration of 0.5 µg/mL (*see* **Notes 3**, **4**, **31**, and **32**).
6. Let the agarose mix cool down before pouring it into the platform (*see* **Notes 33** and **34**).
7. Once the gel is poured, let it harden at room temperature without moving it. Remove the tapes from the ends and carefully remove the comb (*see* **Note 35**).
8. Place the platform in the gel electrophoresis tank, fill the tank with the appropriate buffer to cover the gel, and get rid of the air pockets trapped underneath the gel.
9. Apply the DNA molecular-weight marker (cold and radiolabeled), DNA controls, and DNA samples with a pipetman into the wells by injecting them into the bottom of the well underneath the thin layer of buffer covering the gel (*see* **Notes 36–38**).
10. Attach the lead to ensure the DNA migrates into the gel toward the anode (positive lead). Set the voltage to the desired level, typically 1–10 V/cm of gel. Typically, for an overnight run of a 20-cm gel (recommended for good separation of the TRFs), the voltage is set at 20–30 V (*see* **Note 39**).
11. Turn off the power supply when the bromophenol blue dye from the loading buffer has migrated to an appropriate distance for separation of the desired fragments. If EtBr has not been added to the gel mix, soak the gel in the EtBr bath for 30 s (if your EtBr bath is very concentrated, then, soak the gel for a longer period), then rerun for 10–15 min.
12. DNA in the gel can be visualized by placing it on UV light source (>2500 µW/cm^2) (*see* **Note 40**) and photographed directly using cameras with appropriate filters.

3.1.4. Transfer of the DNA Onto a Nylon Membrane

1. Trim the wells and any unused areas from the gel using a scalpel blade.
2. Depurinate the DNA by soaking the gel for 10 min in depurination solution; leave it in a glass tray at room temperature.
3. Rinse the gel briefly with distilled water.
4. Denature the DNA by soaking the gel in denaturation solution for 1 h.
5. Rinse the gel with distilled water and place it in transfer solution for 15 min.
6. Place three layers of Whatman paper cut the size of the gel and pre-soaked in transfer solution on the gel transfer apparatus (*see* **Note 5**). Get rid of any bubbles trapped between the 3MM papers by rolling a pipet over their surface.
7. Place the gel over these papers and get rid of bubbles as in **step 6**. Place parafilm layers tightly around the gel but do not cover any part of it (*see* **Note 41**).
8. Flood the surface of the gel with transfer solution. Place the pre-cut and pre-wet (in transfer solution) nylon membrane on top of the gel *(see* **Note 42**), and get rid of bubbles (as in **step 6**).
9. Wet two pre-cut 3MM papers in transfer solution and place them on the membrane. Get rid of air bubbles (as in **step 6**).
10. Put a stack (15 cm high) of hand paper towels centered on top of the set up. Put a glass plate on the pile and put a large book centered on top of the glass.
11. Let the alkaline transfer to proceed for at least 4 h (overnight is recommended).
12. Remove the paper towels and the 3MM papers. Recover the membrane, mark the position of the wells, and identify the membrane (*see* **Note 42**).
13. Rinse the membrane briefly in 5X SSC + 0.01% SDS, let the surplus of liquid drain off the membrane, and let it dry at room temperature on towel paper for 15 min.
14. Proceed to the pre-hybridization step or wrap the membrane in plastic wrap and store at 4ºC until further utilization.

3.1.5. Labeling and Purification of DNA Probes

3.1.5.1. Random Prime Labeling of DNA Fragments

1. Resuspend 50–250 ng of desired DNA-fragment in 14 µL of TE, pH 8.0 (*see* **Note 43**).
2. Heat to 100ºC for 5 min.
3. After rapid cool-down, add 11.4 µL LS-buffer, 1 µL of the dATP, dGTP, dTTP mix, 3 µL α^{32}P-dCTP, 1 µL Klenow DNA Polymerase (5 U) (*see* **Note 20**).
4. Incubate at room temperature from 3 h to overnight.
5. Add 30 µL 0.2% SDS, 50 m*M* EDTA, pH 8.0. Incubate at 65ºC for 10 min.
6. Purify the probe from unincorporated nucleotides by G-50 Sephadex column (*see* **Subheading 3.1.5.3.**).

3.1.5.2. 5'-End Labeling of Oligonucleotides

1. Mix: 2 µL of oligonucleotides (100 ng/µL) (*see* **Note 44**); 2 µL 10X T4 polynucleotide kinase buffer; 5 µL γ^{32}P-ATP; 1 µL T4 polynucleotide kinase (10 U); 10 µL H_2O.
2. Incubate at 37ºC for 45 min (*see* **Note 20**).
3. Incubate at 65ºC for 10 min.
4. Add 30 µL TE, pH 8.0, and proceed to purification of the probe from unincorporated nucleotides on a G-50 Sephadex column (*see* **Subheading 3.1.5.3.**).

3.1.5.3. Purification of Probes

1. Resuspend the G50-resin in the column by vortexing.
2. Loosen the cap one-fourth turn and snap off the bottom closure.
3. Place the column in a 1.5-mL microcentrifuge tube for support.
4. Pre-spin the column for 1 min at 735*g* (*see* **Note 45**).
5. Wash the column twice with the appropriate buffer (the buffer in which the probe is resuspended) by loading 50 µL and spinning at 735*g* for 1 min.
6. Place the column in a new 1.5-mL tube and slowly apply 50 µL of the sample to the top-center of the resin, being careful not to disturb the resin bed (*see* **Note 20**).
7. Spin the column at 735*g* for 1 min. The purified sample is collected in the support tube. Store the tube containing the probe at room temperature in a plexiglas rack and behind a radioprotective screen.
8. Probe-specificity determination can be done following the manufacturer's recommendations or the protocol described in **ref. *54***.

3.1.6. Prehybridization and Hybridization

3.1.6.1. Using Random-Prime Labeled Probes

1. Heat the superstock solution to 42ºC and incubate for 1 h before use.
2. Place the membrane in a heat-sealable bag and seal three sides of the bag (*see* **Note 46**). Add the prewarmed superstock to the bag (~0.5 mL/cm^2 of membrane), remove all air bubbles, and seal bag (*see* **Notes 47** and **48**).
3. Place the bag in a water bath at 42ºC for at least 1 h (overnight is recommended).
4. Heat the superstock + dextran solution to 42ºC until complete dissolution (~0.5 mL/cm^2 of membrane). During this time, heat-denature the equivalent of 6×10^6 cpm of radiolabeled probe by boiling it at 100ºC for 5 min.
5. When the dextran sulfate is completely dissolved in the 50-mL Falcon, add the denatured probe and mix well.
6. Cut one corner of the bag with the membrane and drain the superstock solution. Add the superstock + dextan + probe solution and squeeze as many air bubbles as possible out of the bag. Reseal the bag without any air bubbles (*see* **Note 20**).
7. Incubate the bag submerged in a water bath at 42ºC for at least 12 h.

3.1.6.2. Using 5'-End-Labeled Oligo-Probes

1. Place the membrane in a heat-sealable bag and seal three sides of the bag (*see* **Note 43**). Add in-gel hybridization solution (~0.5 mL/cm^2 of membrane) and the radiolabeled probe and seal the bag without air bubbles (*see* **Notes 47** and **48**).
2. Hybridize at 37ºC overnight.

3.1.7. Washing of the Membrane and Autoradiography

3.1.7.1. Washing Conditions for Random-Prime Labeled Probes

1. Recover the hybridization solution from the bag into a new tube, because it is reusable for more hybridization (*see* **Note 49**).
2. Gently remove the membrane from the bag and place it in a plastic container with 10 mL of 2X SSC, rinse quickly, and discard 2X SSC. Pour fresh 2X SSC on the membrane and let the membrane at room temperature on a rocking platform for 20 min.
3. Discard the solution and add 25–50 mL of 0.1X SSC + 0.1% SDS. Place in a water bath at 55ºC for 1 h with agitation.
4. Remove membrane from the solution, verify background radioactivity on the membrane with a hand-held Geiger counter; if too much background is present, rewash the membrane as in **step 3**. Otherwise, seal the membrane in a plastic bag and proceed to autoradiography.
5. Place membrane in a suitable cassette between two signal-amplifying screens (*see* **Note 50**). Make sure the cassette is well-closed and is light-proof. Adequate exposures usually take an overnight in the –80ºC freezer (*see* **Fig. 1B**).

3.1.7.2. Washing Conditions for 5'-End-Labeled Oligo-Probes

1. Remove the hybridization solution from the bag and dispose of it with radioactive waste.
2. Place the membrane in a plastic container with 10 mL 0.25X SSC, rinse quickly, and discard 0.25X SSC. Cover the membrane again with 0.25X SSC and put on a rocking platform at room temperature for 3 h, changing the solution once.
3. Verify the background radioactivity on the membrane with a hand-held Geiger counter; if too much signal is detected, continue washing the membrane either at room temperature or at 30ºC. Otherwise seal the membrane in a heat-sealable bag and proceed to autoradiography.
4. Place membrane in a suitable cassette between two signal-amplifying screens (*see* **Note 50**). Make sure the cassette is well-closed and is light-proof. Adequate exposures usually take an overnight in the –80ºC freezer.

3.1.8. Stripping of the Membrane

1. Incubate the membrane at 45ºC for 30 min in transfer solution.
2. Place the membrane in stripping solution and incubate at 45ºC for 15 min.

3. Monitor the amount of probe left on the membrane by re-exposing it (*see* **Subheading 3.1.7.1.**). If the probe is all gone, repeat the hybridization steps as in **Subheading 3.1.6.**

3.2. Yeast Senescence Assays

3.2.1. Yeast Streaking on Selective Media and Growth of Different Yeast Strains

1. Streak strains to be tested on appropriate media plates (refer to **Subheading 2.1.1.**) using sterilized toothpicks. The streaking technique used should maximize the number of single colonies to be visible on the plate (*see* **Note 51**).
2. Correctly identify the plates (media composition, dates, and the culture growing) on the plate (*see* **Note 52**). Seal the plate with parafilm. Grow the cells at the desired temperature; usually 3–4 d at 30ºC, longer for cells at 23ºC.

3.2.2. Colony Analysis on Plates

1. The plates with successive passages of the studied strain have to be closely observed, because phenotypes of senescence and/or cellular death sometimes are hard to discern (*see* **Notes 53** and **54**).

3.2.3. Appearance of Survivors and Analysis of Their Phenotypes

1. Cultures undergoing senescence display a gradual decrease in the ability to form colonies. Thus, on plates with the third or fourth restreaks (*see* **Fig. 2A,B**), the majority of colonies should be very small and/or of irregular shape after 3–4 d of incubation. However, except for special genetic combinations (*see* **Notes 55** and **56**) there almost invariably will be normal looking colonies on the particular plates. These are called survivors ***(11–14)***. Such cultures are derived from cells that have bypassed the requirement for telomerase and will grow fairly normally thereafter.

3.2.4. Survivor Analysis in Liquid Cultures

1. Yeast colonies are inoculated into appropriate liquid medium (10 mL), grown at desired temperature and diluted in 12–48 h intervals 1:10,000 into fresh medium.
2. The OD at 600 nm (OD_{600}) is taken before and after each dilution and loss of growth is monitored. When reaching senescence, the culture will be unable to divide and the OD_{600} will remain unchanged, even after extended incubations. However, upon further incubation, the OD_{600} of the culture will re-start increasing and the doubling time of the culture may even become faster than before the arrest. This re-growth indicates that the culture has been overgrown by survivors.
3. To examine DNA from individual colonies, cells can be grown in 2 mL of liquid medium followed by TRF analysis via the Southern blot procedure (*see* **Subheadings 2.1.1.–2.1.8.** and **3.1.1.–3.1.8.**).

3.3. Terminal DNA–Structure Analysis

*3.3.1. Yeast Cultures and DNA Preparation (*see ***Subheading 3.1.1.****)*

*3.3.2. DNA Digestion (*see ***Subheading 3.1.2.****)*

3.3.3. DNA Controls

Yeast G-tails can be detected in native gels using a complementary C-rich oligonucleotide probe. As controls for hybridization, G-rich single-stranded DNA (referred to as ssGT) and C-rich single-stranded DNA (referred to as ssCA) are used as positive and negative controls, respectively. Another important control for the native gel is the double-stranded DNA control (referred to as dsDNA). This control is important to make sure no DNA denaturation occurred during the procedure, especially during the drying step.

Removal of G-tails on native telomeres by nuclease treatments, either with Exonuclease I or Mung bean nuclease, is another useful control. This treatment validates that the signals obtained in native conditions are owing to terminal overhangs, and not to internal gaps in the telomeric C-strand. Exonuclease I is the favorite enzyme to validate this point because it is a 3'-end-specific single-stranded DNA exonuclease.

3.3.3.1. Single-Stranded DNA Controls

Single-stranded DNA from plasmids pCA75 (referred as ssCA) and pGT75 (referred as ssGT) were obtained by standard procedures using a helper phage ***(54)***. Approximately 5 ng of ssDNA controls is loaded on the agarose gel.

3.3.3.2. Double-Stranded DNA Control

Plasmid pMW55 is either linearized with either *Eco*RI or *Bam*HI (4.4 kb) or *Pvu*I-digested prior to loading on the gel (1.9 kb and 2.5 kb DNA fragments). The 1.9 kb double-stranded DNA fragment contains the 55 bp of yeast telomeric repeats. Approximately 20 ng of dsDNA control is loaded onto the agarose gel.

The ssGT control (described in **Subheading 3.3.3.1.**) can be mixed with the dsDNA control, *Pvu*I-digested pMW55, and loaded in the same well of the agarose gel.

3.3.3.3. Genomic DNA Treatments

Treatment of genomic DNA by nuclease must be done on intact chromosomes, i.e., before digestion of DNA by restriction enzymes.

3.3.3.3.1. Exonuclease I Treatment

1. In an reaction tube, mix 1–2 µg of genomic DNA, 5 µL of 10X *Exo*I buffer, 50 U of Exonuclease I (final conc. 1 U/µL), and complete to 50 µL with water (*see* **Notes 57** and **58**).
2. Incubate the reaction for 1 h to overnight at 37°C (*see* **Note 59**).
3. Add 100 µL of 2X stop buffer and 50 µL of water.
4. Extract once with phenol/chloroform.
5. Precipitate the DNA by adding 2 volumes of 100% cold ethanol.
6. Wash the DNA pellet with 70% ethanol.
7. Gently resuspend the pellet in 10 µL TE.
8. Digest DNA with an appropriate restriction enzyme (usually *Xho*I) as described in **Subheading 3.1.2.**

3.3.3.3.2. Mung Bean Nuclease Treatment

Mung bean nuclease is a single-strand-specific DNA endonuclease. Therefore, it is not specific for removal of 3' telomeric extensions.

1. In a reaction tube, mix 1–2 µg of genomic DNA, 5 µL of 10X Mung bean buffer, 25 U of Mung bean nuclease (final conc. 0.5 U/µL), and complete to 50 µL with water (*see* **Notes 57** and **58**).
2. Incubate for 10 min at 37°C (*see* **Note 60**).
3. Add 100 µL of 2X stop buffer and 50 µL of water.
4. Extract once with phenol/chloroform.
5. Precipitate DNA by adding 2 volumes of 100% cold ethanol.
6. Wash the DNA pellet with 70% ethanol.
7. Gently resuspend the pellet in 10 µL TE.
8. Digest DNA with an appropriate restriction enzyme (usually *Xho*I) as described in **Subheading 3.1.2.**

3.3.4. Labeling of DNA Probes and Purification

3.3.4.1. 5'-End Labeling of Oligonucleotides (*see* **Subheading 3.1.5.2.**)

3.3.4.2. Random Priming (*see* **Subheading 3.1.5.1.**)

3.3.4.3. Purification of Probes (*see* **Subheading 3.1.5.3.**)

3.3.5. Gel Electrophoresis (see ***Subheading 3.1.3.***)

Also, *see* **Note 61**.

3.3.6. Gel Drying

Drying the gel is the critical step of the in-gel hybridization technique. This procedure should be done carefully and one might have to fiddle around with the gel-dryer setup in order to get the conditions right (*see* **Note 62**).

1. After completion of gel electrophoresis, stain gel with EtBr and take a picture. Then immerse the gel in 2X SSC for 30 min at room temperature (RT), in a glass container.
2. Put gel upside down (i.e., the open wells down) on two layers of Whatman paper and cover it with plastic wrap.
3. Dry the gel on a gel dryer at RT for 12–20 min (*see* **Note 62**).
4. Flip gel over and dry another 12–20 min at RT.
5. Gel should now be very thin and even (*see* **Notes 63** and **64**).

3.3.7. In-Gel Hybridization Procedure

1. Put the gel into a sealable plastic bag. Seal the plastic bag around the gel, keeping 2 cm of free space on one side of the gel for hybridization procedure.
2. Cut an opening in one corner of the sealed bag.
3. Add 15–30 mL of in-gel hybridization solution in the plastic bag, depending on the size of the gel (*see* **Notes 65** and **66**).
4. Look carefully for holes in the sealed bag.
5. Add the 5'-end radiolabeled oligonucleotide probe and mix it with the hybridization solution (*see* **Notes 67** and **68**).
6. Remove any bubbles by the opening on the bag and seal the plastic bag.
7. Hybridize the dried gel at 37°C for 16 h.

3.3.8. Washing Conditions

3.3.8.1. Gel Washing After Hybridization

1. After hybridization, pour off the solution in an appropriate recipient for radioactive waste.
2. Put the gel in a recipient containing 0.25X SSC solution at RT (*see* **Note 69**).
3. Wash the gel at RT for 1.5 h.
4. Pour off the washing solution and add again 0.25X SSC solution.
5. Wash the gel for another 1.5 h.
6. Remove the gel from the 0.25X SSC solution and put the gel in a new plastic bag. Seal the plastic bag.
7. Place the thin gel in an X-ray film cassette with a film and expose properly. Usually, the exposure time required for detection of single-strand DNA is 1–3 d (*see* **Notes 70** and **71**). An example of the results is shown in **Fig. 3**.

3.3.8.2. Probe Removal in Native Conditions

This procedure is useful if the gel needs to be hybridized in native conditions with more than one probe. After hybridization to the first probe, it is removed in native conditions, and the same gel can then be re-hybridized to a new probe.

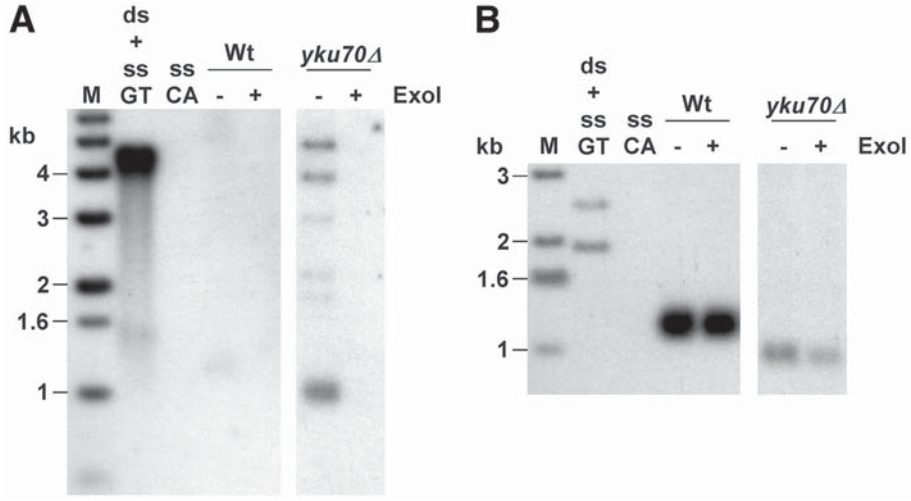

Fig. 3. Detection of telomeric G-tails for Wt and *yku70Δ* yeast cells by in-gel hybridization. **(A)** Genomic DNA isolated from Wt and *yku70Δ* strains was either mock-treated (labeled *Exo*I-), or treated with *E. coli* Exonuclease I (labeled *Exo*I+), before digestion with *Xho*I. The native gel was probed with a ^{32}P-labeled $C_{1-3}A$ oligonucleotide probe (CA-probe) to detect G-tails. Single-stranded phagemid DNA containing yeast telomeric repeats of the G-rich strand (ssGT) and of the C-rich strand (ssCA) serve as a positive and negative control, respectively. The ssGT control was mixed with *Pvu*I-digested pMW55, the latter being the double-stranded control (ds, a fragment of 1.9 kb containing telomeric repeats). Note the presence of strong G-tail signals for DNA derived from *yku70Δ* strain as compared to the one derived from the wt strain, as previously reported ***(19,20)***. **(B)** The same gel was then denatured, transferred onto a membrane by Southern blotting and the blot hybridized to a Y'-probe, detecting TRFs. The procedure shows equal loading of DNA in each lane, thus showing that the strong signal detected in **(A)** is owing to longer single-stranded G-rich tails rather than more DNA being loaded in these lanes.

1. Put the gel in a recipient containing 0.25X SSC solution pre-warmed at 35°C (*see* **Note 72**).
2. Wash the gel at 35°C for 1.5 h.
3. Pour off the washing solution and add again pre-warmed 0.25X SSC solution.
4. Wash the gel for another 1.5 h at 35°C.
5. Remove the gel from the 0.25X SSC solution and put the gel in a new plastic bag. Seal the plastic bag.

6. Place the thin gel in an X-ray film cassette with a film and expose at least 16 h in order to determine if the probe was totally removed from the gel (*see* **Note 73**).
7. Re-hybridized the gel as described in **Subheading 3.3.7.**

3.3.9. Denaturation of DNA and Rehybridization

3.3.9.1. Denaturation of DNA and In-Gel Rehybridization

After required exposures are done on the native gel, the DNA can be denatured in the gel and detected by rehybridization to a specific probe. This procedure can be useful to see if similar amounts of DNA were loaded in each lane of the gel, and therefore it serves to quantify the signal obtained to the total DNA loaded.

1. Put the gel into a glass tray and cover it with 500 mL of denaturing solution for 25 min at RT.
2. Remove denaturing solution and add 500 mL of neutralizing solution.
3. Gently shake the gel for 20 min at RT.
4. Remove the denatured gel and put it into a sealable plastic bag.
5. Proceed to hybridization procedure as described in **Subheading 3.3.7.** and to washing procedure as described in **Subheading 3.3.8.**

3.3.9.2. DNA Transfer by Southern Blotting and Probing

Instead of performing the steps in **Subheading 3.3.9.1.**, DNA can be denatured and transferred from dried gel to a nylon membrane by Southern blotting as described in **Subheadings 3.1.4.**, **3.1.6.**, and **3.1.7.** Using this procedure, a randomly labeled DNA probe can be used for hybridization and multiple rounds of hybridization/washing steps can be performed using different probes (in the in-gel hybridization technique, the gel is more subject to damage and DNA becomes fuzzy after a few weeks). An example of the results is shown in **Fig. 3**.

3.4. Telomere PCR and Sequencing

*3.4.1. Yeast Cultures and DNA Preparation (*see ***Subheading 3.1.1.****)*

Ethanol-precipitated DNA is resuspended in 5 m*M* Tris-HCl, pH 8.0, to obtain DNA concentrations of 100–200 ng/mL.

3.4.2. Tailing of the 3' End

1. Use 0.5-mL tubes and add 8 µL of 1X TdT buffer and 1 µL of genomic DNA (100–200 ng) from a yeast strain harboring the DIA5-1 cassette (or other telomere marker).
2. Heat to 95ºC for 5 min (*see* **Note 74**) and cool rapidly to 4ºC (*see* **Note 75**).
3. To the 9 µL reaction, add 1 µL of tailing mix.
4. Incubate 30 min at 37ºC, then heat-inactivate the enzyme (10 min at 65ºC and 5 min at 94ºC). Proceed immediately to the PCR (*see* **Note 76**).

3.4.3. PCR Amplification of 3'End-Tailed Telomeres

1. Transfer the 10 µL tailing reaction to puReTaq™ Ready-To-Go™ PCR Beads. Add 1 mL of each primer (e.g., the dG_{18}-*Bam*HI and DIA5-1 primers at 20 pmol/µL) and X µL of H_2O (final volume 25 mL; *see* **Note 12**).
2. PCR-amplifications are performed with an initial denaturation at 94°C for 2 min, followed by 45 cycles consisting of 20 s denaturation (94°C), 15 s annealing (62°C) and 20 seconds extension (72°C), and a final extension step of 5 min at 72°C (*see* **Note 77**). Reaction products can be stored at 4°C.

3.4.4. Analysis of PCR Products on Agarose Gel (see ***Subheading 3.1.3.***)

1. Reaction products from the previous PCR are analyzed on a 2–3% TAE-Agarose gel (*see* **Note 78**).
2. The band corresponding to the amplified DNA is gel-excised, isolated using the Qiaquick Gel Extraction Kit protocol from Qiagen. Note that DNA is eluted into 30 mL of elution buffer (EB) buffer (10 m*M* Tris-HCl, pH 8.5; *see* **Note 79**).

3.4.5. Cloning of PCR Products Into pGEM-T Vector

DNA products amplified with Taq DNA polymerase bear 3' unpaired deoxyadenosines at both ends. pGEM-T vectors from Promega, constructed with single 3' T-overhangs at the insertion site are suitable for efficient ligation of Taq PCR products according to the T/A strategy.

1. Cloning of PCR-amplified and purified 3'-tailed telomeres is performed using the pGEM-T Vector System I from Promega following the recommendations of the manufacturer. Incubate the ligation reactions overnight at 4°C in order to get a maximum number of transformants.
2. Competent DH5α bacteria are transformed via a heat-shock procedure with a few microliters of the ligation mixture ***(54)***.
3. After overnight incubation, select white colonies and grow them overnight at 37°C in LB medium with ampicillin.
4. Prepare plasmid DNA (we prefer here the QIAprep Spin Miniprep Protocol from Qiagen). Elute into 30 µL H_2O (*see* **Note 79**).
5. Digest 1 µL of recovered plasmid DNA for verification of the length of the cloned insert (see pGEM-T map for restriction sites), and analyze on a 2% TAE-Agarose gel. A 3 kb band corresponding to the pGEM-T vector and a band of the size corresponding to the cloned telomeric fragment are expected.

3.4.6. DNA Sequencing

Plasmids containing cloned inserts are ready to be sequenced using pUC/M13 forward primer and a SequiTherm EXCEL™II DNA sequencing kit on a LiCor DNA-sequencer. For a valid sequencing result, the following conditions should be met: the sequenced plasmid has to contain on one end 54 bp corresponding to the DIA5-1 primer and on the other end 26 bp derived from dG_{18}-

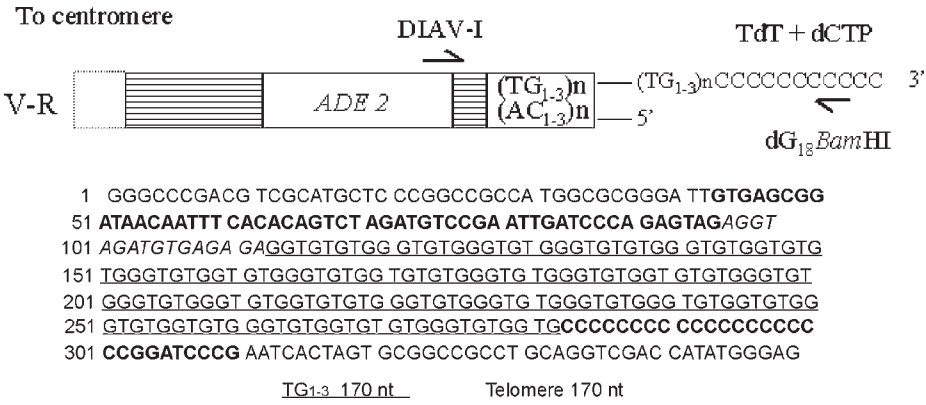

Fig. 4. Example of a telomere sequence obtained with the sequencing protocol. Sequences identified are, from top; in blue, sequence of the DIAV-I primer; black in italics, short stretch of Y'-sequence (as expected in this construct); red, 170 nt of yeast telomeric repeats; blue, oligoC and dG18*Bam*HI primer. The black sequences outside are vector sequences expected from the pGEM-T plasmid.

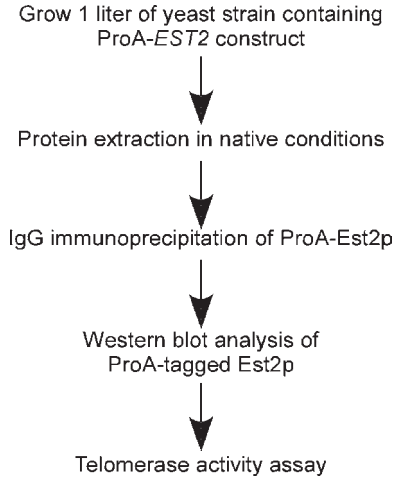

Fig. 5. Overview of the telomerase activity assay.

*Bam*HI; in between the two primer sequences, the tailed-telomeres of various lengths should be recovered. If every requirement is met by the sequence obtained, then the sequencing result can be taken into consideration. An example of the sequencing result is shown in **Fig. 4**.

3.5. Telomerase Assay

3.5.1. Preparation of Yeast Cell Cultures and Protein Extraction

The main characteristic of this assay is to allow a detection of in vitro telomerase activity on a G-rich telomeric primer. However, yeast telomerase is a low-abundance enzyme and not easily assayed. Thus, this method includes an enrichment step for the telomerase enzyme. For this, the strain used as a source of telomerase must have the *EST2* gene tagged at N-terminus with a protein A sequence tag. The tag is used to immunoprecipitate the Est2p protein (the catalytic subunit of yeast telomerase) along with the essential *TLC1* RNA. It is important to note that the ProA-*EST2* construction has been made via various steps as described originally in **ref. *35***. For an overview of the entire procedure for the telomerase assay, *see* **Fig. 5**.

1. Streak desired yeast strain containing ProA-*EST2* construct on YEPD plate for single colonies. Put at 30°C or at the required temperature for 2–3 d or until the colonies are of correct size.
2. Pick a colony and inoculate 5 mL YEPD; grow culture overnight at 30°C (or required temperature).
3. Inoculate 1 L of YEPD media with the 5-mL overnight culture and grow the cells to an OD at 600 nm of approx 1.0.
4. Transfer the cell culture into Beckman 500-mL centrifuge bottles (or equivalent).
5. Centrifuge at 4°C, 3000*g* in Beckman JLA-10.500 rotor, for 5 min (*see* **Note 80**).
6. Rinse the cell pellet one time with ice-cold H_2O. Centrifuge at 4°C, 3000*g* for 5 min.
7. Rinse the pellet one time with 500 mL ice-cold TMG/NaCl. Centrifuge at 4°C, 3000*g* for 5 min.
8. Rinse again with approx 30 mL TMG/NaCl. Spin down in 35-mL Oakridge tubes (VWR 21009-386) 5 min at 3000*g* at 4°C, in Beckman JA-25.50 rotor (or equivalent) (*see* **Note 81**).
9. Resuspend the pellet in 1 pellet-volume (approx 2.5 mL) of TMG/NaCl/DTT containing protease inhibitors.
10. Transfer to 16 × 100-mm glass tubes (this allows for a better lysis with glass beads).
11. Add glass-beads up to about 2 mm below the meniscus.
12. Vortex at maximum speed for 30 s and repeat 25 times (between each step, let stand 30 s on ice). This should be done in a cold room to avoid protein degradation. Visually assess cell lysis by inspecting an aliquot under a light microscope (or phase-contrast); lysis should be at least 80%.
13. Using a large pipet tip, remove the liquid from the glass tubes, and transfer into Eppendorf tubes.
14. Rinse the glass beads two times with 1 mL TMG/NaCl/DTT containing protease inhibitors (briefly vortex each time). In order to remove all the liquid, be sure to poke the pipet tip all the way down the glass tubes (*see* **Note 82**).

15. Pool the corresponding extracts and transfer into 14-mL centrifuge tubes with caps (Sarstedt, cat. no. 55.538, or equivalent).
16. Centrifuge for 15 min at 725*g* at 4°C in a Beckman JA-25.15 or equivalent rotor.
17. Transfer the supernatant to Eppendorf tubes.
18. Centrifuge for 30 min at maximum speed in a cold microcentrifuge.
19. Transfer the supernatant into new tubes and centrifuge for an additional 15 min in cold microcentrifuge.
20. Transfer into Eppendorf tubes in aliquots of 500 µL.
21. Flash freeze on ethanol/dry ice. Store at –80°C.
22. The protein concentration of crude cleared extracts can be measured via a Bradford dosage assay (Bio-Rad). Usually, extracts have concentrations of total protein of approx 3–10 mg/mL.

3.5.2. Immunoprecipitation of ProA-Est2p

3.5.2.1. Preparation of IgG Sepharose Beads

Starting notes: 60 µL of beads (final) are needed for every immunoprecipitation. There will be some losses during washes; thus we recommend preparing 75 µL of beads per immunoprecipitation (*see* **Notes 83** and **84**).

1. Centrifuge the required volume of beads for 1 min at 3000 rpm in a cold microcentrifuge. Remove as much supernatant as possible (*see* **Note 85**).
2. Add 1 mL of TMG buffer A. Incubate for 5 min at 4°C with gentle agitation on LabQuake rotator.
3. Spin for 1 min at 725*g* in cold microcentrifuge and remove the supernatant. Repeat **steps 2** and **3** three more times.
4. Wash the beads once with TMG buffer B (adjust to 0.5% Tween-20) and proceed to the same incubation and spin as in **steps 2** and **3**.
5. Following the final wash, resuspend the beads in one bead-volume of TMG buffer B (with 0.5% Tween-20).

3.5.2.2. Immunoprecipitation

1. Thaw 1 mL of protein extract on ice (or the equivalent of 3–5 mg of total protein). Add 1 µL RNasin and 5 µL Tween-20 (0.5% final).
2. Add 120 µL of 50% slurry IgG beads previously washed to the adjusted protein extract.
3. Incubate at least 4 h or overnight at 4°C, with gentle agitation.
4. Wash the beads twice with 0.5 mL TMG buffer B (0.5% Tween-20). Incubate for 10 min at 4°C. Centrifuge and remove supernatant as abovementioned.
5. Wash the beads twice with 0.5 mL TMG buffer A (add 1 µL of RNasin per 0.5 mL buffer). Incubate, centrifuge, and remove supernatant.
6. Resuspend the beads in 20 µL TMG buffer C (add 1 µL RNasin per 0.5 mL buffer).
7. Aliquot the beads, usually 10 µL per tube, and flash-freeze on dry ice.

3.5.3. SDS-PAGE Analysis of ProA-Est2p

The following description assumes the use of a Hoefer Mighty Small Dual Gel Caster and Hoefer Mighty Small II electrophoresis apparatus. The recipes are described for the making of two mini-gels. Be sure to wash and scrub the glasses carefully and rinse with distilled water and 100% ethanol before use. Ten percent polyacrylamide gels are usually used for analysis of yeast telomerase as the ProA-tagged Est2p weighs about 135 kDa. Recipes for the separating gels can be modified in order to obtain another percentage.

1. 10% separating gel: in a 15-mL Falcon tube, mix 4.0 mL ddH_2O, 3.3 mL 29:1 polyacrylamide mix, 2.5 mL 1.5 *M* Tris, pH 8.8, 100 µL 10% SDS, 100 µL 10% ammonium persulfate (APS) solution, and 4 µL TEMED.
2. Pour the gel in the apparatus, leaving enough space for the stacking gel and comb.
3. Add a small layer of water-saturated isobutanol on top of the gel because this will remove any trace of air bubbles and will prevent evaporation. The gel should let stand to polymerize for about 20–30 min (*see* **Note 86**).
4. Once the gel is polymerized, pour off the isobutanol over a sink and rinse the top of the gel thoroughly with H_2O. Use a paper towel to carefully remove any remaining water.
5. Prepare the stacking gel by mixing 2.7 mL H_2O, 670 µL 29:1 polyacrylamide mix, 500 µL 1.0 *M* Tris, pH 6.8, 40 µL 10% SDS, 40 µL 10% APS, and 4 µL TEMED.
6. Pour the stacking gel on top of the polymerized separating gel and insert the combs. Let polymerize for 20 to 30 min.
7. Prepare the 1X running buffer by diluting 100 mL of the 10X stock with 900 mL of water. Mix.
8. Once the stacking gel is polymerized, mark the emplacement of the wells on the glass with a permanent marker and carefully remove the comb. Transfer the gel into the Hoefer Mighty Small II electrophoresis apparatus and add the running buffer as required.
9. Using a 1-mL syringe, a 22-gauge needle and running buffer, carefully wash the wells to remove chunks of polyacrylamide gel.
10. To the 10 µL of IgG bead suspension (immunoprecipitation products), add 10 µL of 2X Laemmli loading buffer (with freshly added 2-mercaptoethanol, 10% final concentration). Heat for 5 min at 100°C and load. Reserve one lane for 5–10 µL of pre-stained molecular-weight markers, heated prior to loading.
11. Complete the assembly of the gel unit and connect to a power supply. Run the gels at 100–150 V for 1 h or until the bromophenol blue dye runs off the gel. Be sure that the top markers (higher molecular weights) are well-separated because ProA-Est2p is migrating in this region (*see* **Fig. 6A**).

3.5.4. Western Blotting for Detection of ProA-Est2p

Use cold transfer buffer at this step. Electrophoretic transfer is done using a Bio-Rad Mini Trans-Blot Cell tank system. It is important that the assembled

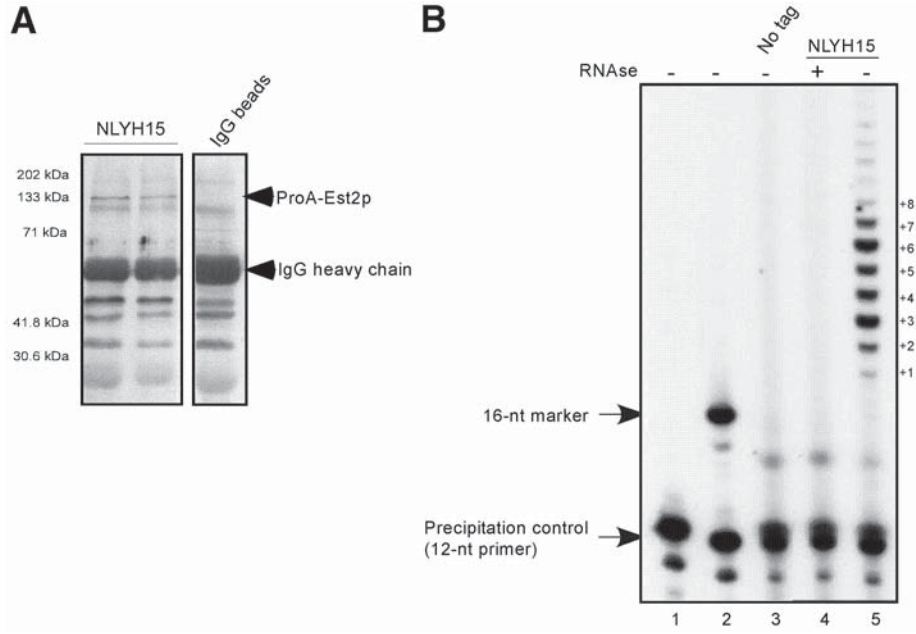

Fig. 6. Western blot of immunoprecipitated ProA-Est2p and in vitro telomerase activity assay. **(A)** Five-min exposure of a Western blot probed with anti-ProA antibody as described in the text. NLYH15, wild-type yeast strain harboring the ProA-*EST2* construct (kindly provided by K. L. Friedman, Nashville, TN). The two lanes represent two different immunoprecipitations. In the third lane, only IgG beads boiled in 10 µL 2X Laemmli loading buffer have been loaded. Note: It is possible to dose the amount of ProA-Est2p with respect to the IgG heavy chain band that lights up when the anti-ProA antibody is used. **(B)** Lane 1, Precipitation control alone (12-nt radiolabeled primer); lane 2, 16-nt marker, which represents the minimum length of the telomerase substrate; lane 3, Immunoprecipitated protein extracts from a yeast strain that does not harbor a ProteinA tag on the EST2 gene. The fourth and fifth lanes represent the actual assay and show immunoprecipitated ProA-Est2p from an otherwise wild-type strain (NLYH15). Lane 4, the immunoprecipitated extracts have been exposed to RNase A. Because telomerase is dependent on its internal RNA, this serves as the characteristic negative control. Lane 5, characteristic pattern of addition of radiolabeled dGTP to the telomeric primer. The gel was exposed for 2 d.

transfer pack eventually is entirely submerged in buffer. In a small bowl containing transfer buffer, place the sheets of Hybond C nitrocellulose membrane (cut just a bit larger than the size of the separating gel). Allow equilibration for about 10 min. Disassemble the electrophoresis apparatus containing the separated samples and follow the procedures below to mount the transfer set-up.

1. Remove and discard the stacking gel from the separating gel. Cut one corner of the separating gel to allow orientation to be followed.
2. Place the transfer cassette into a larger tray containing transfer buffer, such that the negative electrode-side (cathode) is submerged. Put one of the two wet sponges on the cassette.
3. Cut four pieces of 3MM Whatman chromatography paper the same size as the Hybond-C membrane. Place two pieces on the sponge and lay the separating gel on top. The nitrocellulose membrane is then put on top of the gel (cut the same corner as the gel to allow for orientation) and the other two pieces of Whatman paper are applied on the membrane. Using a ruler, remove any trapped air by pressing down on the sandwich; making sure that the entire set-up is submerged in the buffer.
4. Place the other sponge on top of the sandwich and close the transfer cassette. Transfer the cassette into the electrophoresis tank (*see* **Note 87**).
5. Fill the tank with cold transfer buffer. Place the apparatus in a cold room and attach to power supply. A magnetic stir-bar is placed into the tank and activated to allow circulation and cooling of the buffer. The transfer is done for 2 h at 85 V or overnight at 30 V.
6. Once the transfer is complete, remove the cassette from the tank. Carefully disassemble the transfer set-up. Remove the nitrocellulose membrane from the gel and place it in a recipient suitable for blocking (*see* **Notes 88** and **89**). The gel can be discarded or stained with Coomassie brilliant blue.
7. The nitrocellulose membrane is then incubated for at least 1 h in approx 25 mL cold blocking solution on a rocking platform (*see* **Note 90**).
8. Remove the blocking solution and wash the membrane three times with cold PBS-T (10 min each wash) with vigorous shaking.
9. The primary antibody, polyclonal anti-Protein A, is then applied to the membrane. Use a dilution of 1:10,000 in 10 mL antibody dilution solution (1% milk + PBS). Incubate membrane with primary antibody for 1 h at room temperature on a rocking platform.
10. Remove primary antibody solution (*see* **Note 91**) and perform three washes of the membrane as described in **step 8**.
11. Freshly prepare the secondary antibody solution: we routinely use a 1:5000 dilution in antibody dilution solution (1% milk + PBS). Incubate membrane with the diluted secondary antibody for 1 h at room temperature on a rocking platform.
12. Wash the membrane three times, as in **step 8**. During the final wash, you can prepare the ECL reagents (e.g., let them warm up to room temperature).
13. Place the membrane on a sheet of plastic wrap that you had previously fixed onto your bench-top.
14. Apply the ECL mix (mix reagent A with reagent B in equal volumes, 1 mL is necessary to cover one mini-gel size membrane) and let stand for 1 min on the membrane. Be sure that the membrane is completely covered. After 1 min, the excess ECL solution is removed by lifting one corner of the membrane with tweezers and touching the opposite corner of the membrane on a paper towel (membrane vertical).

15. Place the membrane between two transparent plastic sheets (could be a cut hybridization bag or acetate sheet protectors) and place into an X-ray film cassette. Be sure to mark the orientation of your membrane with any fluorescent or luminescent tape.
16. In a dark room, place a Bio-Max Blue XB-1 film in the cassette and expose for suitable exposure times (*see* **Note 92**). An example of the result is shown in **Fig. 6B**.

3.5.5. In Vitro Telomerase Activity Assay

Once the native enzyme is enriched via immunoprecipitation, it can be assayed. Note that during the whole procedure, it is important to avoid any RNase contamination, because RNases may degrade the *TLC1* RNA, which is an essential element for telomerase activity.

3.5.5.1. Preparation of the 16-nt Marker

In a separate lane of the gel, unreacted and end-labeled 16-nt primer will be loaded. Thus, in a separate tube, label the 5'-end of the telomeric primer with T4 polynucleotide kinase (T4 PNK; *see* **Subheading 3.1.5.2.**). It will serve as the starting point of nucleotide addition by the telomerase (+0).

3.5.5.2. Preparation of the 20% Acrylamide/8 *M* Urea Sequencing Gel

1. For general information on setting up sequencing gels, *see* **ref. *54***.
2. For one long sequencing gel, mix 50 mL of the 20% acrylamide/8 *M* urea ready mix with 400 µL 10% APS and 30 µL TEMED. Using a 50-mL syringe, pour the gel between the two glass plates without making bubbles. Sqeeze in the comb last and wait until the gel has polymerized (~30 min).
3. Pre-run the gel 45 min at 40 W, then load the samples on the gel, using 1X TBE buffer in the gel apparatus.

3.5.5.3. In Vitro Telomerase Activity Assay Using Immunoprecipitated ProA-Est2p

1. Spin 10 µL immunoprecipitation beads at 725*g* for 1 min and remove the 5 µL supernatant to leave only 5 µL packed beads.
2. Add 0.5 µL H_2O or 0.5 µL RNase (10 mg/mL). Adding RNase in one tube will serve as a negative control to show that telomerase activity is abolished owing to degradation of *TLC1* RNA (*see* **Note 93**).
3. Incubate 10 min at 30°C, and place on ice.
4. To each reaction tube, add the following: 1.4 µL telomeric primer oligo (TAGGGTAGTAGTAGGG), 1.1 µL nucleotide mix (1 m*M* each, no dGTP), 1.5 µL master mix, 1.5 µL (α-^{32}P) dGTP (*see* **Note 20**). Incubate for 20 min at 30°C.
5. Add 1.5 µL Stop buffer and 1.75 µL Proteinase K (20 mg/mL) to the tubes and incubate for 30 min at 65°C.
6. Add 1 µL of radiolabeled 12-nt oligo, diluted to approx 3000 cpm/µL and 85 µL H_2O (*see* **Note 94**).

7. Add 100 µL phenol/chloroform, vortex, spin 5 min, and transfer the aqueous phase (top) to new microcentrifuge tubes (*see* **Note 95**).
8. Add 66 µL of 5 *M* ammonium acetate and 1 µL 20 mg/mL glycogen to aqueous phase. Precipitate DNA with 500 µL 100% cold ethanol. Place at –20°C for at least 1 h.
9. Spin for 30 min at maximum speed in a microcentrifuge, at 4°C.
10. Remove the supernatant and wash the pellet with 1 mL of cold 70% EtOH. Spin for 10 min at 4°C, maximum speed.
11. Very carefully and without disturbing the pellet, remove the ethanol with a pipet. The pellet can easily detach from the tube. Dry the pellet and resuspend in 4 µL formamide loading buffer.
12. Prepare two new tubes for the marker and precipitation controls. To do so, mix 1 µL of labeled 16-nt marker (diluted at 3000 cpm/µL) in 3 µL formamide loading dye. Do the same with 1 µL of labeled precipitation control (3000 cpm).
13. Before loading, heat the samples for 2 min at 100°C.
14. Run the 20% acrylamide/8 *M* urea sequencing gel for approx 2.5 h, at 40 W (*see* **Note 96**).
15. Disassemble the gel apparatus carefully; avoid breaking the gel. Transfer gel onto a Whatman paper by reversing the gel, which remained stuck to one of the glasses during disassembly, and applying it onto a paper (*see* **Note 97**). Once the gel is transferred, cover it with a plastic wrap avoiding the formation of bubbles. Place into an X-ray film cassette of the appropriate size.
16. In a dark room, place a film of the appropriate size on the gel and expose for at least 2 d at –80ºC. We usually expose for 2–5 d. See **Fig. 6B** for an example of a telomerase assay.

4. Notes

1. Hoechst dye is a possible mutagen. Wear gloves when handling and wear a mask when weighing.
2. All solutions must be at room temperature before measuring fluorescence. Prepare assay solution fresh when ready to use. Filter TNE buffer before adding dye. Do not filter once dye is added.
3. Ethidium bromide is a powerful mutagen and is moderately toxic. Wear gloves and lab coat when handling. After use, the solutions and gels should be safely eliminated, according to your institution's safety measures for toxic waste.
4. Always put the acid into the water and NOT THE CONTRARY.
5. If the transfer apparatus is not already made, you can set one up this way:
   a. Choose a tray that can accommodate the size of the gel (large pyrex container).
   b. Choose a glass or plastic support that is longer and wider than the gel (sequencing glass-plates work well).
   c. Place the support on the tray so that it overlaps and is supported by the ends of the tray.

d. Cut 2 sheets of Whatman 3MM paper long enough to overhang the support and place them on the support so the hanging parts dip into the tray.
e. Fill the tray with transfer solution until the level of the liquid reaches almost the top of the support.
f. Cut parafilm to cover this set-up and cover the whole apparatus with plastic wrap.
g. Always take off the plastic wrap and the parafilm before using the set up and recover after the use to minimize evaporation.

6. A denatured fragment of double-stranded DNA can also be used.
7. DNA preparations do not have to be very clean; RNase A and proteinase K treatments can be skipped. Nevertheless, better results are obtained with the DNA extraction procedures described in **Subheading 3.1.1.**
8. The RWY12 strain has a unique subtelomeric region located on the right arm of chromosome V (VR-*ADE2*-T ***[24]***), which allows a specific amplification with the DIA5-1 primer. In order for the PCR reaction to be specific, yeast strains have to harbor such a specific marker on one telomere ***(17)***.
9. We recommend using 5X TdT buffer from Gibco instead of 10X One-Phor-All Buffer *PLUS* (Amersham Biosciences). The presence of cobalt (Co^{2+}) in the reaction buffer helps the tailing of any type of 3'-end.
10. Prepare a master mix according to the number of reactions to be performed. Example, for 10 reactions use 0.5 µL TdT enzyme at 20 U/µL, 2 µL 5X TdT buffer, 1 µL dCTP at 10 m*M*, and 6.5 µL H_2O.
11. Quality of DNA oligo-synthesis may vary depending on companies. Gibco primers are good enough for this PCR-based method, but Forstemann et al. recommend using DNA oligos from MWG ***(28)***.
12. When a bead is reconstituted to a 25 µL final volume, each reaction contain approx 2.5 U of puReTaq™ DNA polymerase, 10 m*M* Tris-HCl, pH 9.0, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 µ*M* of each nucleotide (dATP, dCTP, dGTP, and dTTP), and stabilizers, including BSA.
13. Alternatively, a conventional PCR mix buffer can be used as described in **ref. *28***. If this option is chosen, follow tailing and telomere PCR methods from **ref. *28***. TdT, like most enzymes, is inhibited in the presence of EDTA. Therefore, DNA has to be dissolved in an EDTA-free solution in order to allow the 3' end polymerization reaction.
14. This is a neurotoxin when unpolymerized. Use special care when handling (e.g., wear protective gloves).
15. You can heat the gel mix to help dissolve the urea before pouring the gel, but note that this procedure will seriously decrease the polymerization time (i.e., it can polymerize when you are pouring it and therefore you will have to redo the setting and the gel mix). Also, a 20% polyacrylamide denaturing gel is used to separate adequately fragments ranging from 10–100 nucleotides.
16. Various primers have been used for this assay; the one indicated here has been shown to give the best results.

17. We use an oligo- primer with the sequence $(T_2AG_3)_2$ as an internal control for precipitation.
18. Technically, only TTP is needed because yeast telomerase only adds G and T nucleotides.
19. Wear gloves and a lab coat while working with phenol, because it is a very dangerous compound. Also, use polypropylene tubes when working with phenol. The nucleic acid will tend to partition into the organic phase if the phenol has not been adequately equilibrated to a pH of 7.8–8.0. Normally, the aqueous phase forms the upper phase. However, if the aqueous phase is dense because of the presence of salts or sucrose, it will form the lower phase. The organic phase is easily identifiable because of the yellow color given by the hydroxyquinoline added during the phenol equilibration. You should always dispose of phenol waste in a specially sealed container and ensure that it is eliminated according to your establishment's politics for dangerous wastes.
20. Working with radioactivity is dangerous and should be taken seriously. Always wear a lab coat, two pairs of gloves, and work behind protective screens. Verify often that your hands and the materials used are not contaminated by direct verification using a hand-held Geiger counter, and make a complete verification of your work-space when the manipulation is completed.
21. You should always be working in a sterile environment (around a Bunsen burner), because yeast cultures are easily contaminated by bacteria and other airborn fungi.
22. You should flame your tube before opening and work near a flame during your inoculation. Incline your tube for inoculation with cells; the media will mount along the side of the tube, which makes it easy to gently rub the toothpick and the colony into the media, while staying in a sterile environment. After the inoculation, flame-sterilize the top of the tube again before putting on cap.
23. If the used culture tubes are in good conditions (no cracks or breaches) and they can withstand centrifugation, you can use them directly for this centrifugation step. However, glass tubes can be fragile and break easily in the centrifuge with concomitant loss of the culture. Therefore, it is preferable to transfer the overnight culture into a plastic tube (e.g., 15-mL Falcon tubes) before centrifugation.
24. Be careful about which tubes you use. If the volume is too big for the tube, vortexing will be less effective. Also, the use of glass tubes is recommended, because it will allow efficient cell lysis with the glass beads.
25. It is important not to put too much glass bead in the tubes to allow the liquid to move during the vortexing steps and ensure an efficient cell lysis. Also, it is important to always leave the tubes on ice between the vortexing steps to minimize the possible action of diverse proteases released in the mix.
26. Move the pipet tip through the glass bead slurry to the bottom of the tube and recover a maximum of cells. It does not matter if you carry a few glass beads into the microcentrifugefuge tubes; you will discard them in the next steps.
27. The protocol we use is an adapted method from **ref. *55***.
28. For final DNA concentrations between 100–500 µg/mL, use 1 µg/mL Hoechst in 1X TNE.
29. Accuracy in pipetting is critical for reproducible results. A pipetman accurate to 0.02 µL is recommended.

30. Bal 31 is an exonuclease that degrades DNA from a double-strand end inwards. Therefore, sensitivity to this enzyme is used extensively to assess the terminal location of telomeric sequences.
31. Handle the boiling solution very carefully. Swirl gently to allow overheated solutions to cool down.
32. Although possible, we do not recommend adding the EtBr directly to the gel solution. Gels should be stained after electrophoresis in an EtBr-containing solution before taking a photograph.
33. Pouring the gel mix too hot may cause the casting platform to break, especially at the sealed joints.
34. When pouring the mix, make sure the casting platform is on a flat surface. Also, make sure no bubbles are trapped underneath the comb and remove all bubbles on the surface of the gel by using either a Kleenex® or a pipet tip.
35. If the gel is not hard enough, it might be useful to put it at 4ºC for a couple of minutes to accelerate the process. Be cautious when removing the comb, because the gel may sometimes be very fragile and the wells can easily be cracked.
36. The cold DNA molecular-weight marker is complemented with radiolabeled DNA molecular weight marker before loading on the gel. This will allow detection of the marker by EtBr staining as well as on the membrane and the autoradiogram. Usually, radiolabeled 1 kb DNA molecular-weight marker is diluted to 20,000 cpm/µL, and 1 µL of this diluted mix is added to 19 µL of cold marker.
37. We recommend loading the two most exterior lanes of a gel with the radiolabeled 1 kb marker, which allows an assessment of the even migration of the DNA in all lanes of the gel.
38. Appropriate controls (single-stranded CA sequences, single-stranded GT sequences, etc.) should be loaded in separate wells of the gel to allow detection by the radiolabeled probe to be used afterwards.
39. To prevent electrical shocks, the gel apparatus should always be covered and the power supply shut down before handling gels.
40. UV light is damaging to the eyes and exposed skin. Protective eyewear should be worn at all times while using a UV light source.
41. The parafilm serves as a barrier to prevent liquid from flowing directly from the reservoir to paper towels placed on top of the gel.
42. It is recommended to wear gloves and use blunt-ended forceps to manipulate the membrane. Avoid touching the membrane directly with hands.
43. DNA fragment used as telomeric probe is a 300 bp fragment containing 286 bp of yeast telomeric repeats from pYLPV ***(15)***. A heat-denatured 600 bp *Kpn*I-*Kpn*I fragment of Y' sequences ***(8)*** cloned in the *Kpn*I site of pVZ1 ***(51)*** was used as Y' control.
44. The oligos used as probes are described in **Subheading 2.3.7.**
45. It is important to calculate the speed at which the column should be centrifuged. The following formula can be used: rpm = (1000) $(657/r^{1/2}$, where r = radius in mm from center of spindle to bottom of rotor bucket and rpm = revolutions per minute. Also, the column should be used immediately after preparation to avoid drying out of the resin.
46. Check the bag for leaks before adding radiolabeled probes. To do so, verify that there

are no slow leaks (drops) when you squeeze the solution up to the sealed sides.

47. It is easy to get rid of bubbles by gently rubbing the membrane on a corner such as the end of a table. If needed, you can seal a little amount of solution out of the bag to get rid of all bubbles.
48. After this step, it is important that the membrane is not allowed to dry, because this will cause problems for the hybridization and washing steps.
49. To reuse the superstock + dextran + probe solution, boil the entire mix at 100ºC for 5 min and put it in the bag containing the membrane that had been pre-hybridized.
50. The use of screens is to amplify the signal obtained. They are not useful unless you put the cassette at –80ºC. They should be placed on the exterior sides of the membrane and the film.
51. The first streak serves to deposit the cells on the plate and is done with one side of the round-end of a toothpick. The toothpick is discarded and a new streak is initiated in the dense cell deposit and then streaking on the plate to obtain single cell colonies.
52. The writing of notes must be done underneath the plate to avoid mixing up when changing plate covers. The bottom of the plate can also be separated in quadrants to allow the streaking of many strains on the same plate.
53. The appearance of senescent phenotypes as well as survivor phenotypes also can be monitored via the use of Southern blots (*see* **Subheadings 2.1.1.–2.1.8.** and **3.1.1.–3.1.8.**) with genomic DNA extracted from yeast cells grown for different numbers of generations. During successive passages of senescent cells, a shortening of telomeres can be observed, and as the cells enter a survivor mode, the pattern of TRFs will resemble those of either type I or II (*see* **Fig. 1C**).
54. The number of generations before the appearance of senescence can vary depending on the deletion or the background of the strain, but usually occurs after 80–100 generations. Assuming that a healthy 2-mm colony contains about 2×10^6 cells, there are about 20 generations needed to obtain such a colony from a single deposited cell. This means that for a strain lacking any of the yeast telomerase components, senescence should be visible after 4–5 restreaks (*see* **Note 55**).
55. Combining the deletion of any of the yeast telomerase genes with a deletion of the *RAD52* gene will abolish the appearance of survivor cells. Such cells will also senesce faster, usually after 20–40 generations (2–3 restreaks, *see* **Fig. 2B** and *see* **Note 56**).
56. You can obtain a double-mutant strain (+ your particular condition) in two ways. Either start with a diploid cell that is heterozygous for the deleted alleles and after sporulation, select the double-mutant haploid by selection of the spores on appropriate restrictive media for the deleted alleles. Alternatively, use haploid cells harboring the two deletions and also containing the *TLC1* gene on a *URA3* plasmid to allow viability, which is easily selected against by streaking the cells on FOA media. Cells growing on such plates then are real double-mutants and can be assayed for senescence.
57. When assembling reactions, always add enzyme last. Removal of telomeric 3' overhang is more efficient when performed on clean genomic DNA. If results are

unsatisfactory, genomic DNA extracted by standard glass bead or NIB procedures can be purified over a Sephadex G-50 column as previously described ***(54)***.

58. Single-stranded circular DNA, e.g., M13mp18 phage DNA, can be used as a control for endonuclease activity. Usually, we use 300 ng of M13mp18 phage DNA (Amersham Biosciences) in the reaction. Single-stranded and double-stranded DNA can be used also as positive controls for the endonuclease activity test.
59. Usually, we treat yeast genomic DNA with Exonuclease I for 1–2 h at 37°C.
60. Alternatively, the reaction can be performed at 30°C for 30 min.
61. For the in-gel hybridization technique, we generally use 0.75% agarose gels run in 1X TBE. Gels of higher agarose concentrations are hard to dry and subject to rehydration during the hybridization procedure. On the other hand, lower concentration agarose gels are easier to dry, but small DNA fragments could be lost during the drying step.
62. It is recommended to test different drying conditions with your own gel dryer using a small gel containing only the radiolabeled ladder, as control. After migration, take a picture of the gel to see the different bands of the ladder. Dry the gel until it gets as thin as plastic wrap and expose it directly to film at –80ºC. The autoradiogram should allow you to clearly see the 500 bp, and even the 400 bp, as seen on the EtBr picture of the gel. If you do not see these bands, it means that the gel was over-dried, and you will lose the small DNA fragments. When you clearly see those bands, you can also verify if the gel was dried enough by putting it in a bag with the in-gel solution for 1 h at 37ºC. If the gel swells, it was not dried enough and the drying conditions should be optimized (because this will cause problems when you use a radiolabeled probe).
63. After drying, the gel should be a bit thicker than 3MM paper, but not as thin as plastic wrap. When the gels are not dried enough, the gel can re-hydrate during the hybridization step. However, when the gels are over-dried, DNA fragments smaller than approx 1 kb tend to be blotted out instead of staying in the gel (*see* **Note 62**).
64. Upon removal, the gel should stick to the plastic wrap. If not, just slightly wet the Whatman papers with 2X SSC and then remove gel.
65. For a gel measuring14 cm × 14 cm, we use about 20 mL of hybridization solution.
66. In order to determine if the gel is dried enough, incubate the gel with the in-gel hybridization solution at 37°C for at least 1 h. If the gel remains thin, the probe can be added to the solution. However, if the gel rehydrates, re-dry the gel until it becomes a bit thinner than 3MM paper.
67. We use a total of 10–20 ng of radiolabeled probe per gel for hybridization, yielding about $1–2 \times 10^6$ cpm total per gel.
68. For an efficient hybridization, we use oligonucleotides between 20–30 nucleotides long as probes.
69. For less stringent conditions, the gel can be washed with 0.25X SSC washing solution at lower temperatures (e.g., 4°C). Smaller telomeric overhangs can be more efficiently detected by this washing procedure.
70. If the result obtained is a spotted gel, cleaning the probe should resolve the problem for the next gel. The spots are probably caused by aggregation of probe in the

gel matrix. It is almost impossible to get rid of such spots, but rewashing the gel at a slightly higher temperature (e.g., at 30°C) might greatly improve the result.

71. If the result obtained is heavy background, it is probably caused by rehydration of the gel during the hybridization procedure. To get rid of the background, freeze the gel at –80ºC and let thaw at room temperature. Remove the excess water from the bag and re-expose at –80ºC. Repeat the procedure 3–4 times. The gel should be washed for at least 1 h in 0.25X SSC at RT before re-exposure.
72. Usually, the probes are easily removed from the gel when it is washed at 35°C or higher. Thus, depending of the length of the oligonucleotide used as a probe and the length of overhangs present on DNA, the washing temperatures should be between 35°C and 50°C.
73. If the probe remains hybridized to DNA molecules, wash the gel again at higher temperatures with 0.25X SSC for 2X 1.5 h.
74. Tailing reaction can be performed directly on a yeast colony, then allow 10 min reaction at 95ºC for denaturation.
75. This step increases tailing efficiency by disrupting the possible secondary structures formed owing to the high percentage of G residues in the telomere sequence.
76. Tailed DNA is not easily stored (Forstemann and Lingner, personal communication); we suggest performing the PCR step just after the tailing reaction.
77. Optimization of telomere PCR conditions should be done according to thermal cycler used.
78. Nice gels of that percentage without bubbles and foam at the surface can be obtained by adding 5 µL of 10% Triton X-100 to 50 mL TAE/Agarose slurry before boiling. (Be patient, such mixes boil over very easily. Forstemann and Lingner, personal communication).
79. With DNA dissolved in water, better sequencing profiles are obtained. However, the pH should be around 8.5 for an efficient elution of DNA from the QIAprep column.
80. Everything must be kept on ice (or in a cold room) because proteins are easily degraded by proteases when extracts are left at room temperature.
81. The cell pellet can be flash frozen at this step and stored at –80°C.
82. It does not matter if beads are taken up with the liquid because they will be removed in further steps.
83. Usually, 50% slurry of beads and buffer is the starting solution. To obtain a final 60 µL of beads, you should pipet 120 µL of the 50% slurry. You also have to pipet a little more to compensate for the losses of beads during the washing steps.
84. It is easier to pipet the beads using cut pipet tips.
85. In order to remove as much liquid as possible, a 1-mL syringe with a mini needle can be used such that the beads stay in the tube and the liquid is removed.
86. Excess gel can be kept in the Falcon tube to use as a polymerization index.
87. Usually, the transfer cassettes are colored to place them in the correct orientation. The membrane should be on the side of the positive electrode (between the gel and the anode). It is very important to make sure that this orientation is correct or the proteins will be lost from the gel into the buffer instead of transferring to the nitrocellulose membrane.

88. Staining the membrane with Ponceau Red solution can check if the transfer is correct (without bubbles). The solution is completely washed out of the membrane during further steps.
89. On the membrane, the molecular-weight markers should be clearly visible.
90. In order to decrease background problems caused by the primary antibody, blocking can be extended to overnight at 4°C.
91. Keep the antibody-containing solution at –20°C for a maximum two other uses.
92. In order to detect ProA-Est2p, exposing for 5 min is usually enough. However, longer exposures may be needed, depending on the quality of immunoprecipitation and Western blotting procedures. *See* **Fig. 6A** for a typical Western blot for ProA-Est2p after a 5-min exposure.
93. This will also tell if the bands that appear on the final film are really owing to telomerase activity.
94. This serves as the precipitation control. The oligo is radiolabeled using (γ-^{32}P) ATP and the 5'-end labeling protocol as described.
95. Care should be taken to not take up beads that are at the interface because they might interfere with the running of the acrylamide gel.
96. After 2 h, check the position of the lower Bromophenol Blue dye (it co-migrates with DNA fragments of sizes around 8 nucleotides). It should not go out of the gel.
97. The gel can be left on one of the glass plates and exposed as is. Put a plastic wrap on the gel and place it in an X-ray film cassette. Also, if an old film of the appropriate size is available, it can be used to transfer the gel on it. The gel easily sticks to the film and the transfer is done easily afterwards. Again, a plastic wrap should be applied on the gel without making bubbles.

Acknowledgments

We thank all past and present members of the Wellinger lab as well as S. AbouElela for discussions and help with the development of some of the techniques described here. We also thank J. Lingner for communicating tips on the telomere sequencing technique. Work in our laboratory is supported by grants of the Canadian Institutes of Health Research (CIHR, MOP12616) and the Canadian Cancer Society. C.L. was supported by a studentship of NSERC, M.L. by an MRC studentship, and RJW is a Chercheur-National of the Fonds de Recherche en Santé de Québec (FRSQ).

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24

Controlled Expression of Recombinant Genes and Preparation of Cell-Free Extracts in Yeast

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Summary

Biochemistry is an important experimental tool in the study of protein functions. Biochemical studies frequently involve overexpression of a cloned gene and purification of the recombinant protein. The yeast *Saccharomyces cerevisiae* provides an effective system for expression and purification of recombinant proteins owing to the ease of applying molecular techniques and obtaining large quantities of cells with a low cost. Additionally, complex biochemical processes such as transcription and DNA repair can be studied in yeast cell-free extracts in vitro, which benefit greatly from a large collection of well-defined mutant strains. Controlled gene expression and preparation of cell-free extracts are important techniques in the yeast system. Two commonly used inducible gene expression systems, the *GAL1* promoter and the *CUP1* promoter, are described. Protocols of preparing yeast whole cell extracts and nuclear extracts are presented, each of which is designed for specific applications.

Key Words: Gene expression; gene overexpression; protein overproduction; recombinant gene; cell extracts; cell-free extracts; yeast extracts; protein purification; in vitro transcription; in vitro DNA repair.

1. Introduction

Biochemistry is a powerful, and often the only, experimental tool to elucidate the activity of a protein or protein complex. Frequently, biochemical studies involve overexpression of a cloned gene and purification of the recombinant protein. The yeast *Saccharomyces cerevisiae* offers an important system for expression and purification of recombinant proteins. Favorable factors contributing to the use of yeast as an expression system include the followings. First, standard molecular techniques can be easily applied to yeast. Second, large quantities of yeast cells can be obtained. Third, thousands of yeast

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

deletion mutant strains are available for protein expression in various genetic backgrounds. Lastly, setting up the yeast expression system is relatively easy and inexpensive.

After successful overexpression of a recombinant gene in yeast cells, cell-free extracts need to be prepared before any protein purification steps can be carried out ***(1–4)***. Additionally, complex biochemical processes such as transcription and DNA repair may be retained in specially prepared yeast cell-free extracts ***(5–10)***. These elaborately prepared cell-free extracts have been extensively used to study molecular mechanisms of various complex biochemical pathways ***(5–15)***. Sometimes, it may be desirable to analyze protein–protein interactions. In addition to the widely used yeast two-hybrid system ***(16)***, co-immunoprecipitation from yeast extracts offers an alternative approach to study protein–protein interactions. By combining biochemistry with molecular biology and genetics, yeast represents a powerful experimental system for the understanding of a variety of biological functions. Expressing a recombinant gene and preparation of cell-free extracts are important techniques in the yeast system. In this chapter, two systems of inducible gene expression in yeast will be presented, followed by several protocols for preparing yeast cell-free extracts for various biochemical applications.

2. Materials

2.1. Inducible Expression From the GAL1 *Promoter*

1. Yeast expression plasmid vectors pEGU, pEGT, and pEGL (**Fig. 1A–C**) were derived from YEplac195, YEplac112, and YEplac181 ***(17)***, respectively. These vectors can be amplified in *Escherichia coli* using ampicillin resistance as the plasmid selection and contain the 2 µm origin for multiple plasmid replication in yeast cells ***(17)***. In addition to the yeast-inducible *GAL1* promoter, pEGU, pEGT, and pEGL contain the *URA3* gene, the *TRP1* gene, and the *LEU2* gene, respectively, for plasmid selection in yeast cells. Vectors pEGUtag, pEGTtag, and pEGLtag (**Fig. 1D–F**) were derived from pEGU, pEGT, and pEGL, respectively, for N-terminal tagging of the recombinant protein. The tag can be the His_6, HA, c-myc, or GST.
2. YP medium: 2% Bacto-peptone, 1% yeast extract. YP medium is made to 90% volume and sterilized by autoclave.
3. YPD medium: 2% Bacto-peptone, 1% yeast extract, 2% dextrose. YPD medium is made by mixing YP medium and 1/10 volume of sterile 20% dextrose.

Fig. 1. *(opposite page)* Yeast-inducible expression vectors driven by the *GAL1* promoter. Expression from the *GAL1* promoter is induced by galactose. **(A–C)** Untagged vectors; **(D–F)** tagged vectors, in which the N-terminus of the recombinant protein is fused to a tag such as His_6, HA, c-myc, or GST. The cloning sites are indicated inside the box.

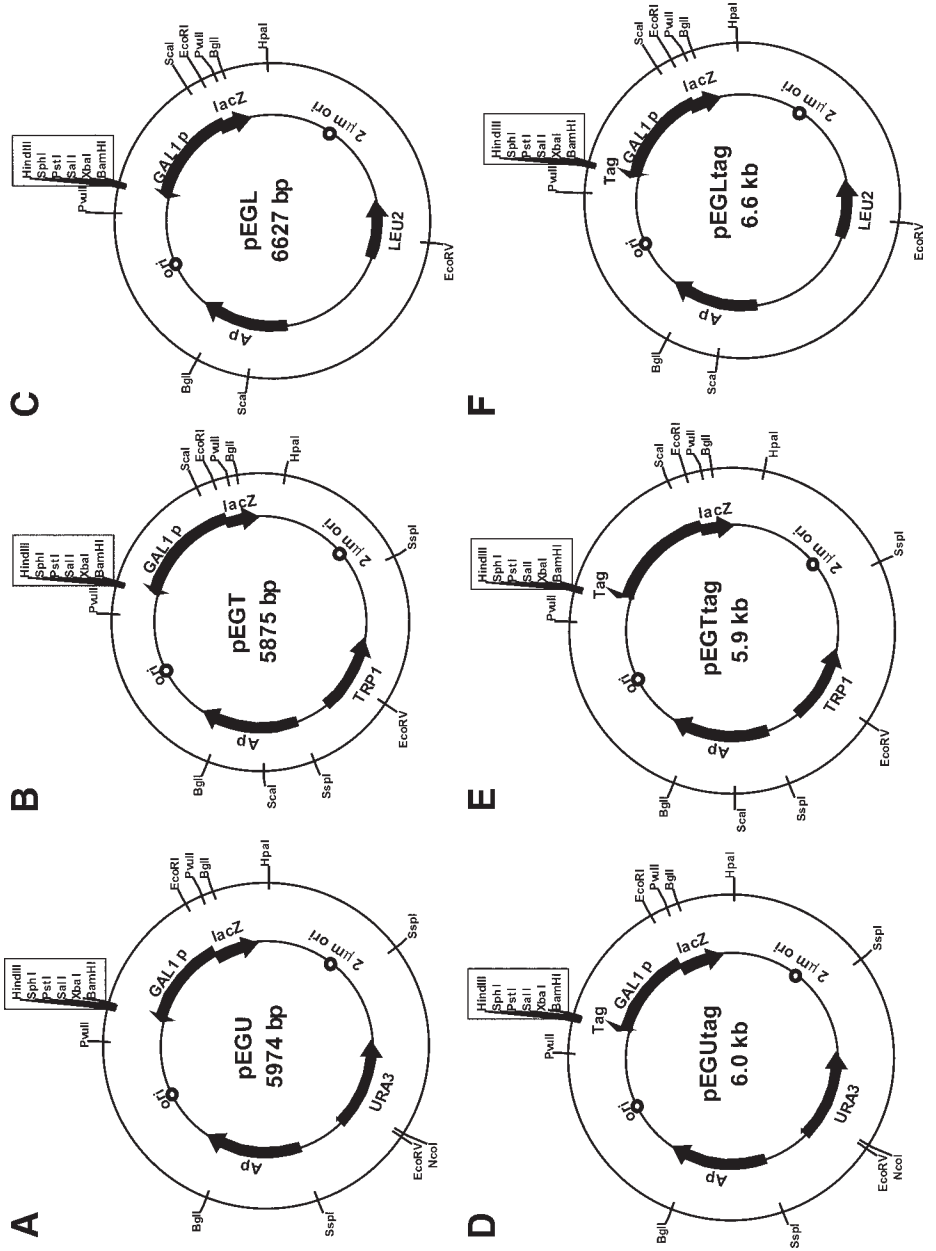
A
pEGU
5974 bp
GAL1 p
lacZ
2 µm ori
ori
Ap
URA3
HindIII
SphI
PstI
SalI
XbaI
BamHI
PvuII
EcoRI
BglII
HpaI
SspI
EcoRV
NcoI
B
pEGT
5875 bp
TRP1
ScaI
C
pEGL
6627 bp
LEU2
D
pEGUtag
6.0 kb
Tag
E
pEGTtag
5.9 kb
F
pEGLtag
6.6 kb

Fig. 1.

4. Minimum medium (YNB medium): 0.17% Bacto-yeast nitrogen base (without ammonium sulfate) and 0.46% ammonium sulfate. YNB medium is made from 10X stock solution that have been sterilized by autoclave. Based on the genotype of the strain, the required amino acids are also added to supplement the medium.
5. Minimum plates: minimum medium containing 1.8% agar. Agar is made in water to 80% of the final volume and sterilized by autoclave. Before use, the agar is melted in a microwave oven and cooled to approx 60°C. YNB medium and dextrose are then added from the respective sterile 10X stock solutions. Based on the genotype of the strain, the required amino acids are also added to supplement the medium.
6. Dextrose: 20% solution as 10X stock and sterilized by autoclave.
7. Sucrose: 20% solution as 10X stock and sterilized by autoclave.
8. Galactose: 20% solution as 10X stock and sterilized by autoclave.

2.2. Inducible Expression From the CUP1 Promoter

1. Yeast expression plasmid vectors pECU, pECT, and pECL (**Fig. 2A–C**) were derived from pEGU, pEGT, and pEGL, respectively, by replacing the *GAL1* promoter with the *CUP1* promoter. These vectors can be amplified in *E. coli* using ampicillin resistance as the plasmid selection and contain the 2 µm origin for multiple plasmid replication in yeast cells. In addition to the yeast-inducible *CUP1* promoter, pECU, pECT, and pECL contain the *URA3* gene, the *TRP1* gene, and the *LEU2* gene, respectively, for plasmid selection in yeast cells. Vectors pECUtag, pECTtag, and pECLtag (**Fig. 2D–F**) were derived from pECU, pECT, and pECL, respectively, for N-terminal tagging of the recombinant protein. The tag can be the His_6, HA, c-myc, or GST.
2. Yeast culture media and plates: *see* **Subheadings 2.1.2.–2.15.**
3. $CuSO_4$: stock solution is made as 100 m*M* and sterilized by filtering though a sterile 0.2-µm membrane.

2.3. Yeast Extracts for Examination of Protein Expression

1. Protease inhibitors: 1 m*M* phenylmethylsulfonyl fluoride (PMSF); 300 mg/mL benzamidine; and 1 mg/mL each of antipain, chymostatin, leupeptin, and pepstatin. Protease inhibitors are prepared as a 100X mixture in ethanol and stored at –20°C. Benzamidine and PMSF are weighed and dissolved directly in ethanol. Antipain, chymostatin, leupeptin, and pepstatin are prepared as 10 mg/mL stock in H_2O, dimethyl sulfoxide (DMSO), H_2O, and methanol, respectively. When needed, protease inhibitors are added to buffers just before use.

Fig. 2. *(opposite page)* Yeast-inducible expression vectors driven by the *CUP1* promoter. Expression from the *CUP1* promoter is induced by Cu^{2+}. **(A–C)** Untagged vectors; **(D–F)** tagged vectors, in which the N-terminus of the recombinant protein is fused to a tag such as His_6, HA, c-myc, or GST. The cloning sites are indicated inside the box. The *Xba*I site in the cloning region is not unique in the plasmid.

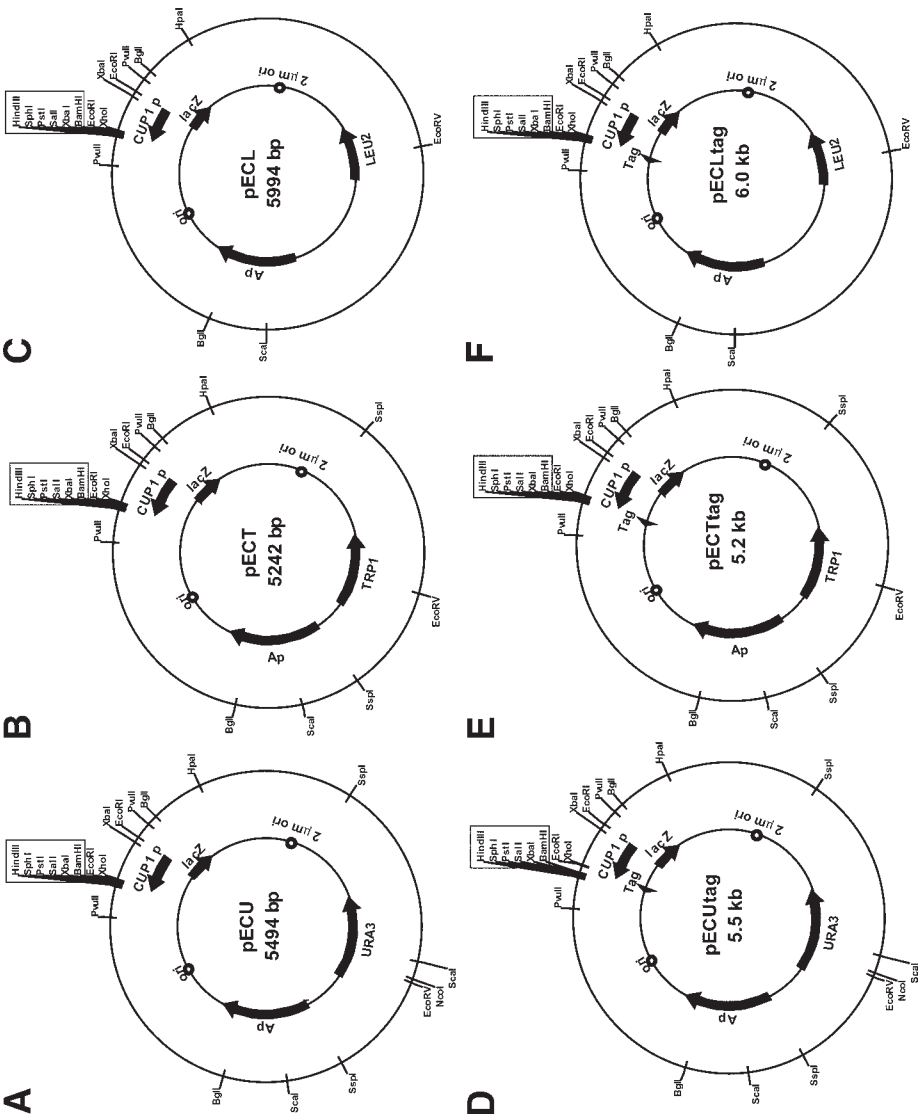
A
pECU
5494 bp
CUP1 p
lacZ
2 µm ori
URA3
Ap
ori
B
pECT
5242 bp
TRP1
C
pECL
5994 bp
LEU2
D
pECUtag
5.5 kb
Tag
E
pECTtag
5.2 kb
F
pECLtag
6.0 kb
HindIII
SphI
PstI
SalI
XbaI
BamHI
EcoRI
XhoI
PvuII
BglI
HpaI
SspI
ScaI
EcoRV
NcoI

Fig. 2.

2. Extraction buffer A: 50 m*M* Tris–HCl, pH 7.5, 0.6 *M* NaCl, 10% sucrose, 5 m*M* β-mercaptoethanol, and protease inhibitors as described in **Subheading 2.3.1.**
3. Zirconium beads: 0.5 mm (Biospec Products, Bartlesville, OK). The beads need to be washed in detergent (such as the Alconox detergent), rinsed in water, and dried before use. Used beads can be re-used following detergent wash, rinse, and drying.
4. Forty percent acrylamide solution (acrylamide:bis-acrylamide, 37.5:1): a 500-mL solution is prepared with 194.8 g of acrylamide and 5.2 g of *N*,*N*'-methylene-bis-acrylamide in distilled and deionized water. The solution is filtered though a 0.2-µm filter and stored at 4°C.
5. Stacking buffer (4X): 0.5 *M* Tris-HCl, pH 6.8, 0.4% sodium dodecyl sulfate (SDS). Store at 4°C.
6. Separating buffer (4X): 1.5 *M* Tris-HCl, pH 8.8, 0.4% SDS. Store at 4°C.
7. SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer (5X): 250 m*M* Tris-HCl, pH 6.8, 2.5% SDS, 358 m*M* β-mercaptoethanol, 0.1% bromphenol blue, and 25% glycerol.
8. SDS-PAGE running buffer (5X): 125 m*M* Tris base, 1.25 *M* glycine, 0.5% SDS.
9. Transfer buffer: prepare a 10X stock solution containing 250 m*M* Tris base and 1.9 *M* glycine. To make 1X transfer buffer, dilute the stock solution by 10-fold and add methanol to 20% and SDS to 0.01%.
10. Wash buffer: prepare a 10X stock solution containing 100 m*M* Tris-HCl, pH 7.5, and 10% NaCl. To make 1X wash buffer, dilute the stock solution by 10-fold and add Tween-20 to 0.05%.
11. BCIP solution: dissolve 100 mg 5-bromo-4-chloro-3-indolyl phosphate in 3 mL of *N*,*N*-dimethylformamide (DMF).
12. NBT solution: dissolve 250 mg of nitro blue tetrazolium by mixing it with 3.5 mL of DMF and 1.5 mL of distilled and deionized water.

2.4. Yeast Extracts for Protein Purification

1. Extraction buffer A: *see* **Subheading 2.3.2.**
2. Zirconium beads: *see* **Subheading 2.3.3.**

2.5. Yeast Whole-Cell Extracts for In Vitro DNA Repair

1. YPD medium: *see* **Subheading 2.1.3.**
2. ED solution: 0.1 *M* ethylenediaminetetraacetic acid (EDTA)-KOH, pH 8.0, and 10 m*M* dithiothreitol (DTT). Store at 4°C.
3. YPS solution: 2% Bacto-peptone, 1% yeast extract, and 1 *M* sorbitol.
4. PMSF solution: 100 m*M* in ethanol. Store at –20°C.
5. Hypotonic buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 5 m*M* DTT, and protease inhibitors (*see* **Subheading 2.3.1.**).
6. Sucrose solution: 50 m*M* Tris-HCl, pH 8.0, 10 m*M* $MgCl_2$, 2 m*M* DTT, 25% sucrose, and 50% glycerol.
7. Neutralized 4 *M* $(NH_4)_2SO_4$: 4 *M* $(NH_4)_2SO_4$, adjust pH to 7.0 using 10 *N* NaOH. Store at room temperature.

8. Dialysis buffer A: 20 m*M* HEPES-KOH, pH 7.6, 10 m*M* $MgSO_4$, 10 m*M* ethylene glycol-*bis*(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA), 5 m*M* DTT, 20% glycerol, and protease inhibitors (*see* **Subheading 2.3.1.**).

2.6. Yeast Whole-Cell Extracts for In Vitro Transcription

1. YPD medium: *see* **Subheading 2.1.3.**
2. Extraction buffer B: 200 m*M* Tris-HCl, pH 7.5, 0.39 *M* $(NH_4)_2SO_4$, 10 m*M* $MgSO_4$, 20% glycerol, 1 m*M* EDTA, 1 m*M* DTT, and protease inhibitors (*see* **Subheading 2.3.1.**).
3. Dialysis buffer B: 20 m*M* HEPES-KOH, pH 7.5, 10 m*M* $MgSO_4$, 10 m*M* EGTA, 5 m*M* DTT, 20% glycerol, and protease inhibitors (*see* **Subheading 2.3.1.**).

2.7. A Yeast Nuclear Extract

1. YPD medium: *see* **Subheading 2.1.3.**
2. ED solution: 0.1 *M* EDTA-KOH, pH 8.0, and 10 m*M* DTT. Store at 4°C.
3. YPS solution: 2% Bacto-peptone, 1% yeast extract, and 1 *M* sorbitol.
4. PMSF solution: 100 m*M* in ethanol. Store at –20°C.
5. Ficoll buffer: 5 m*M* Tris-HCl, pH 7.4, 20 m*M* KCl, 2 m*M* EDTA-KOH, pH 7.4, 0.125 m*M* spermidine, 0.05 m*M* spermine, 1% thiodiglycol, 18% Ficoll, and protease inhibitors (*see* **Subheading 2.3.1.**).
6. Lysis buffer: 100 m*M* Tris-acetate, pH 7.9, 50 m*M* potassium acetate, 10 m*M* $MgSO_4$, 2 m*M* EDTA, 3 m*M* DTT, 20% glycerol, and protease inhibitors (*see* **Subheading 2.3.1.**).
7. Neutralized 4 *M* $(NH_4)_2SO_4$: 4 *M* $(NH_4)_2SO_4$, adjust pH to 7.0 using 10 *N* NaOH. Store at room temperature.
8. Dialysis buffer A: 20 m*M* HEPES-KOH, pH 7.6, 10 m*M* $MgSO_4$, 10 m*M* EGTA, 5 m*M* DTT, 20% glycerol, and protease inhibitors (*see* **Subheading 2.3.1.**).

3. Methods

3.1. Inducible Expression From the GAL1 Promoter

1. Clone the gene of interest into an appropriate vector (**Fig. 1**) under the control of the yeast *GAL1* promoter.
2. Transform yeast cells with the expression construct. Yeast cells grown to an OD_{600} of 2.0 in 50 mL of YPD medium are collected by centrifugation at 3,000*g* for 5 min and washed with sterile water. Cell pellet is resuspended in 1 mL of 100 m*M* sterile lithium acetate and incubated for 10 min at 30°C. Cells are collected by centrifugation and resuspended in 400 µL of 100 m*M* sterile lithium acetate. Cell suspension is divided into 50-µL ($\sim 1 \times 10^8$ cell) aliquots in 1.5-mL microcentrifuge tubes. After brief centrifugation at a low speed, each cell pellet is resuspended in 240 µL of 50% sterile polyethylene glycol (PEG) 3,350 by vortex, and the following components are added: 36 µL of 1 *M* sterile lithium acetate, 5 µL of single-stranded carrier DNA (10 µg/µL) (*see* **Note 1**), plasmid construct, and sterile water to a final volume of 351 µL. After incubation at 30°C

for 30 min, cells are heat-shocked at 42°C for 20 min. Cells are collected by centrifugation in a microcentrifuge for 30 s and then resuspended in 1 mL of sterile water. Approximately 100 µL of the transformed cells are plated on minimal medium plates, followed by incubation at 30°C for approx 3 d.
3. Grow yeast cells containing the expression plasmid construct at 30°C in minimum medium containing 2% sucrose with shaking (~150–200 rpm).
4. When the culture reaches an OD_{600} of approx 1, dilute the culture 10-fold in YP medium. Then, add galactose and sucrose to a final concentration of 2% and 0.5%, respectively (*see* **Note 2**). For small-scale protein expression, 50–100 mL of culture are normally sufficient. For large-scale protein production, as is the case for protein purification, a 2-L culture can be grown in a 6-L flask. Eight flasks (16-L culture) may be required to yield approx 100 g of cell paste for protein purification.
5. Expression of the recombinant protein is induced from the plasmid construct by growing the culture for 12 to 16 h at 30°C with shaking (~150–200 rpm).
6. Collect cells by centrifugation at 4°C, and then wash cells in water. Cell pellet can be stored at –80°C before preparation of cell-free extracts for further analysis or protein purification.

3.2. Inducible Expression From the CUP1 Promoter

1. Clone the gene of interest into an appropriate vector (**Fig. 2**) under the control of the yeast *CUP1* promoter.
2. Transform yeast cells with the expression construct as in **Subheading 3.1., step 2**.
3. Grow yeast cells containing the expression plasmid construct at 30°C in minimum medium containing 2% dextrose with shaking (~150–200 rpm).
4. When the culture reaches an OD_{600} of approx 1–2, dilute the culture 10-fold in YPD medium. Cell growth is continued at 30°C for 6 h with shaking (~150–200 rpm). For small-scale protein expression, 50–100 mL of culture are normally sufficient. For protein purification, 8–16 L of the culture may be needed. Each 2-L culture can be grown in a 6-L flask.
5. Add $CuSO_4$ to a final concentration of 0.3 m*M* to the culture (*see* **Note 3**). Expression of the recombinant protein is induced from the plasmid construct by growing the culture for another 3 h at 30°C with shaking (~150–200 rpm).
6. Collect cells by centrifugation at 4°C, and then wash cells in water. Cell pellet can be stored at –80°C before preparation of cell-free extracts for further analysis or protein purification.

3.3. Yeast Extracts for Examination of Protein Expression

1. Resuspend cell pellet collected from 50 to 100 mL yeast culture in 800 µL of extraction buffer A.
2. Transfer the cell suspension to a 1.5-mL microcentrifuge tube. Then, fill the tube with Zirconium beads such that the cell suspension is filled to the top of the tube. Close the cap, avoiding air bubbles in the tube.
3. Homogenize cells at 4°C in a Mini-Beadbeater (Biospec Products, Bartlesville, OK), using five pulses of 2 min each with a 2-min pause between pulses.

4. Transfer the supernatant to another pre-chilled 1.5-mL microcentrifuge tube, and centrifuge at 20,000*g* (~14,000 rpm) for 15 min at 4°C in a microcentrifuge. Save the cleared supernatant in fresh tubes.
5. Measure protein concentration of the extract. First, prepare a protein standard curve. Prepare standard samples containing 0 (serving as the blank) to 15 µg of pure bovine serum albumin (BSA) in 800 µL of distilled and deionized water each. Then, add 200 µL of the Bio-Rad Protein Assay Dye Reagent Concentrate (Rio-Rad Laboratories, Hercules, CA). After mixing and incubating at room temperature for 5–10 min, OD_{595} is measured in a spectrophotometer. The standard curve is prepared by plotting OD_{595} values against protein concentration (1–15 µg/mL). To measure protein concentration of the yeast extract, 1 µL of the extract, 799 µL of distilled and deionized water, and 200 µL of the Bio-Rad Protein Assay Dye Reagent Concentrate are mixed and incubated at room temperature for 5–10 min. The blank is similarly prepared by mixing 200 µL of the Dye Reagent Concentrate with 800 µL of water. After measuring OD_{595}, protein concentration of the extract is obtained from the protein standard curve. If protein concentration of the yeast extract is greater than 15 mg/mL, dilution is needed before measuring its concentration. A concentration of 8–20 mg/mL is expected for the extract. The extract can be stored at –80°C.
6. Expression of the recombinant protein is determined by Western blot analysis (*see* **Note 4**). Yeast extract of 50–80 µg protein is mixed with 5X SDS-PAGE loading dye and appropriate amount of water. After heating at 90–100°C for 10 min, the sample is loaded onto a 10 or 15% SDS-polyacrylamide gel for protein separation, using a Bio-Rad protean II mini-gel system. The gel consists of the top stacking gel (1.5 cm high) and the bottom separating gel. Electrophoresis is performed in the SDS-PAGE running buffer at constant 150 V, until the dye migrated close to the bottom of the gel, which takes about 45 min to 1 h. The separating gel is sandwiched in transfer buffer as filter paper-nitrocellulose membrane-gel-filter paper, using the Bio-Rad Mini Trans-Blot Cell system. Protein transfer from the gel to the membrane is performed by electrophoresis at constant 400 mA for 45 min. After washing three times in wash buffer, the nitrocellulose membrane is soaked for 5 min in 5% nonfat milk (1 g of dry milk in 20 mL of wash buffer). The membrane is then washed five times in wash buffer, followed by addition of the primary antibody in 10 mL of wash buffer and incubation for 2 h at room temperature or overnight at 4°C. After washing five times in wash buffer, the membrane is incubated with the secondary antibody (alkaline phosphatase-conjugated anti-IgG antibody) in 10 mL of wash buffer. Finally, the membrane is washed five times in wash buffer, followed by addition of 33 µL of BCIP solution and 66 µL of NBT solution in 10 mL of wash buffer. Color development is achieved by incubation at room temperature, and is stopped by washing the membrane in water.

3.4. Yeast Extracts for Protein Purification

1. Collect cells from 8–16 L of culture by centrifugation in a Beckman JA-10 rotor at 4000*g* (6000 rpm) for 10 min at 4°C. Wash cells by resuspending pellets in

water followed by centrifuging as before. Approximately 100 g of cells are expected.
2. Resuspend cells in 150 mL of extraction buffer A.
3. Fill a 300-mL bead-beater (Biospec Products) container with Zirconium beads to one-third full. Completely fill the container with cell suspension, avoiding air bubbles in the container.
4. Homogenize cells on ice in a bead-beater, using 15 pulses of 30 s each with a 2 min pause between pulses.
5. Centrifuge the supernatant at 100,000*g* (33,000 rpm) for 2 h at 4°C in a Beckman type 50.2Ti rotor. Save the cleared supernatant.
6. Measure protein concentration of the extract (*see* **Subheading 3.3., step 5**). After saving a small aliquot for post-purification analysis, the rest of the extract is used for protein purification.

3.5. Yeast Whole-Cell Extracts for In Vitro DNA Repair

This extract was originally developed for in vitro nucleotide excision repair ***(8)***. It also supports in vitro transcription ***(8)***.

1. Grow yeast cells in 500 mL of YPD medium at 30°C with shaking (150–200 rpm) (*see* **Note 5**) to an OD_{600} of 1–3.
2. Harvest cells by centrifugation at 4000*g* (6000 rpm) for 10 min at 20°C in a Beckman JA-10 rotor. Wash once by resuspending cells in water, transferring to a 250-mL Beckman centrifuge bottle, and centrifuging in a Beckman JA-14 rotor for 10 min at 3500*g* (6000 rpm) at 20°C. Weigh the empty bottle before centrifugation and weigh the bottle with the cell pellet again after centrifugation to obtain wet cell weight.
3. Add ED solution at 10 mL/g of cells and resuspend the cell pellet. Incubate at 30°C with shaking (100 rpm) for 10 min. Collect cells by centrifugation as before. Completely remove ED solution (*see* **Note 6**). Remove the last drop with a pipet and wipe bottleneck with Kimwipes.
4. Add YPS solution at 1 mL/g of cells and resuspend the cell pellet. Add yeast lytic enzyme at 1.4 mg enzyme/g of cells (*see* **Note 7**). Incubate at 30°C with shaking (100 rpm) for 1–2 h to convert yeast cells to spheroplasts. Monitor cell-wall digestion under a microscope by adding several drops of water to a drop of cell suspension on a slide. Digestion is complete when approx 90% of cells become spheroplasts that rapidly burst into cell debris in water (*see* **Note 8**).
5. Stop enzyme digestion by adding ice-cold YPS solution at 10 mL/g of cells. Collect cells by centrifugation in a Beckman JA-14 rotor for 10 min at 3500*g* (6000 rpm) at 4°C. From this step, all centrifugations are performed at 4°C and all other procedures are performed on ice.
6. Wash twice in YPS solution by resuspending cell pellet at 10 mL/g of cells and centrifuging. PMSF solution is added to 0.5 m*M* before the last centrifugation. Then, wash once in 1 *M* sorbitol by resuspending cell pellet at 10 mL/g of cells and centrifuging.

7. Weigh the bottle to determine wet spheroplast weight. Resuspend spheroplast pellet in hypotonic buffer to 4 mL/g of spheroplasts. Incubate on ice for 20 min.
8. Transfer the suspension to an ice-cold beaker set in an ice container. Add cold sucrose solution dropwise while stirring gently to 4 mL/g of spheroplasts.
9. Measure the volume and then add neutralized 4 *M* $(NH_4)_2SO_4$ dropwise to 0.9 *M* (290 µL of 4 *M* ammonium sulfate/mL cell suspension) while stirring gently. Stirring is continued on ice for 30 min.
10. Centrifuge the suspension at 170,000*g* for 1 h at 4°C. Pre-chill the rotor and the centrifuge before use.
11. Carefully transfer the supernatant with a pipet to a cold beaker, leaving some residual supernatant behind such that the pellet is not disturbed.
12. Measure the volume of the supernatant and then slowly add solid $(NH_4)_2SO_4$ to 0.35 g/mL over a 20–30 min period with gentle stirring. Neutralize the solution with 10 µL of 1 *N* NaOH/g of $(NH_4)_2SO_4$ added. Continue gentle stirring for another 30 min.
13. Collect protein precipitates by centrifugation at 25,000*g* (18,000 rpm) for 15 min at 4°C in a Beckman JA-20 rotor.
14. Carefully remove the supernatant as much as possible with a pipet. Dissolve protein pellet in 1/30 volume of the ultracentrifugation supernatant in dialysis buffer A. Dialyze the solution overnight at 4°C against dialysis buffer A.
15. Remove protein precipitates by centrifugation at 15,000*g* for 15 min at 4°C. Save the cleared supernatant.
16. Measure protein concentration of the extract (*see* **Subheading 3.3., step 5**). The extract is stored at –80°C and can be frozen and thawed many times without detectable loss of repair activity.

3.6. Yeast Whole-Cell Extracts for In Vitro Transcription

This extract was originally developed for in vitro transcription ***(10)***. It also supports in vitro nucleotide excision repair ***(9)***.

1. Grow yeast cells in 1 L of YPD medium at 30°C with shaking (150–200 rpm) to an OD_{600} of approx 2 (*see* **Note 5**).
2. Harvest cells by centrifugation at 4000*g* (6000 rpm) for 10 min in a Beckman JA-10 rotor. Wash cells in ice-cold water. Then, wash cells in cold extraction buffer B.
3. Scrape cell pellet into a syringe. Then, extrude cell paste directly into liquid nitrogen. Frozen droplets of cell paste may be stored at –80°C.
4. Grind frozen cell pellets under liquid nitrogen using a ceramic mortar and pestle until the material is reduced to powder.
5. Allow liquid nitrogen to evaporate. Then, add 1 volume of cold extraction buffer B. Thaw the mixture at 4°C. Transfer the mixture to a cold beaker. Stir gently for 30 min on ice.
6. Centrifuge the cell lysate at 120,000*g* for 2 h at 4°C.
7. Carefully transfer the supernatant with a pipet to a cold beaker, leaving some residual supernatant behind such that the pellet is not disturbed.

8. Measure the volume of the supernatant and then slowly add solid $(NH_4)_2SO_4$ to 2.94 *M* by adding 337 g/mL over a 30-min period with gentle stirring. Neutralize the solution with 10 µL of 1 *N* NaOH/g of $(NH_4)_2SO_4$ added. Continue gentle stirring for another 30 min.
9. Collect protein precipitates by centrifugation at 25,000*g* (18,000 rpm) for 15 min at 4°C in a Beckman JA-20 rotor.
10. Dissolve protein pellet in dialysis buffer B (~50 µL/g of cells). Dialyze the solution overnight at 4°C against dialysis buffer B.
11. Remove protein precipitates by centrifugation at 15,000*g* for 15 min at 4°C. Save the cleared supernatant.
12. Measure protein concentration of the extract (*see* **Subheading 3.3., step 5**), and store extract at –80°C.

3.7. A Yeast Nuclear Extract

This extract supports base excision repair, nucleotide excision repair, and transcription in vitro ***(14,18,19)***.

1. Grow yeast cells in 2 L of YPD medium at 30°C with shaking (150–200 rpm) to an OD_{600} of 1–3 (*see* **Note 5**).
2. Harvest cells by centrifugation at 4000*g* (6000 rpm) for 10 min at 20°C in a Beckman JA-10 rotor. Wash once by resuspending cells in water, transferring to a 250-mL Beckman centrifuge bottle, and centrifuging in a Beckman JA-14 rotor for 10 min at 3500*g* (6000 rpm) at 20°C. Weigh the empty bottle before centrifugation and weigh the bottle with the cell pellet again after centrifugation to obtain wet cell weight. Approximately 20–40 g of cells are expected.
3. Add ED solution at 10 mL/g of cells and resuspend the cell pellet. Incubate at 30°C with shaking (100 rpm) for 10 min. Collect cells by centrifugation as before. Completely remove ED solution (*see* **Note 6**). Remove the last drop with a pipet and wipe bottleneck with Kimwipes.
4. Add YPS solution at 1 mL/g of cells and resuspend the cell pellet. Add yeast lytic enzyme at 1.4 mg enzyme/g of cells (*see* **Note 7**). Incubate at 30°C with shaking (100 rpm) for 1–2 h to convert yeast cells to spheroplasts. Monitor cell-wall digestion under a microscope by adding several drops of water to a drop of cell suspension on a slide. Digestion is complete when approx 90% of cells become spheroplasts that rapidly burst into cell debris in water (*see* **Note 8**).
5. Stop enzyme digestion by adding ice-cold YPS solution at 10 mL/g of cells. Collect cells by centrifugation in a Beckman JA-14 rotor for 10 min at 3500*g* (6000 rpm) at 4°C. From this step, all centrifugations are performed at 4°C and all other procedures are performed on ice.
6. Wash twice in YPS solution by resuspending cell pellet at 10 mL/g of cells and centrifuging. PMSF solution is added to 0.5 m*M* before the last centrifugation. Then, wash once in 1 *M* sorbitol by resuspending cell pellet at 10 mL/g of cells and centrifuging.

7. Resuspend spheroplasts at 5 mL/g of cells in Ficoll buffer. Lyse spheroplasts in a motor-driven Teflon-glass homogenizer with 10 strokes at 4°C.
8. Centrifuge the lysate in a Beckman JA-20 rotor at 3800*g* (7000 rpm) for 10 min. Centrifuge the supernatant for 5 min at 3800*g* two to four times. After each centrifugation, transfer the supernatant with a pipet to an ice-cold tube. At the last centrifugation, only trace amounts of pellet should be formed.
9. Harvest nuclei by centrifugation at 18,000*g* (15,000 rpm) for 30 min in a Beckman JA-20 rotor. Discard supernatant. The nuclei pellet may be stored at –80°C overnight.
10. Resuspend yeast nuclei at 0.6 mL/g of original cell weight in lysis buffer.
11. Measure the volume and then add neutralized 4 *M* $(NH_4)_2SO_4$ dropwise to 0.9 *M* (290 µL of 4 *M* ammonium sulfate/mL cell suspension) while stirring gently. Stirring is continued on ice for 30 min.
12. Centrifuge the suspension at 170,000*g* for 1 h at 4°C. Pre-chill the rotor and the centrifuge before use.
13. Carefully transfer the supernatant with a pipet to a cold beaker, leaving some residual supernatant behind such that the pellet is not disturbed.
14. Measure the volume of the supernatant and then slowly add solid $(NH_4)_2SO_4$ to 0.35 g/mL over a 20–30-min period with gentle stirring. Neutralize the solution with 10 µL of 1 *N* NaOH/g of $(NH_4)_2SO_4$ added. Continue gentle stirring for another 30 min.
15. Collect protein precipitates by centrifugation at 13,000*g* (13,000 rpm) for 15 min at 4°C in a Beckman JA-20 rotor.
16. Carefully remove the supernatant as much as possible with a pipet. Dissolve protein pellet in 1/20 volume of the ultracentrifugation supernatant in dialysis buffer A. Dialyze the solution overnight at 4°C against dialysis buffer A.
17. Remove protein precipitates by centrifugation at 15,000*g* for 15 min at 4°C. Save the cleared supernatant.
18. Measure protein concentration of the extract (*see* **Subheading 3.3., step 5**). The extract is stored at –80°C and can be frozen and thawed many times without detectable loss of repair activity.

4. Notes

1. Before use, the carrier DNA needs to be denatured by heating at 90°C for 5 min and then chilling immediately on ice.
2. Because the diluted culture already contains 0.2% sucrose, add only 0.3% additional sucrose to give a final concentration of 0.5%.
3. Some strains may exhibit a different sensitivity to $CuSO_4$. Therefore, a preliminary experiment may be needed to determine the tolerable $CuSO_4$ concentrations for a specific strain.
4. If a protein-specific antibody is not available, an epitope tag will be very useful in protein detection. In this regard, the His_6 tag is widely used, because it can be used for both protein detection and affinity-protein purification.

5. Temperature-sensitive strains can be grown at the permissive temperature. If a strain containing a plasmid is used, cells should be grown in minimum medium to maintain plasmid selection.
6. It is essential to completely remove ED solution. Residual ED solution is a strong inhibitor of yeast lytic enzyme.
7. Digestion of yeast cells by yeast lytic enzyme is a critical step. We have tested several different preparations of yeast lytic enzyme from ICN, and found that the enzyme preparation supplied at 70,000 U/g gave the best results.
8. Cells grown in minimum medium or cells grown to the stationary phase are more resistant to digestion by yeast lytic enzyme. Therefore, the enzyme amount should be doubled to digest the cell wall of these cells.

Acknowledgments

The author would like to thank R. Daniel Gietz for the yeast plasmids YEplac112, YEplac195, and YEplac181. This work was supported by NIH grants CA67978 and CA92528.

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25

Production of Heterologous Proteins in Yeast With the Aid of the Hsp150Δ Carrier

Marja Makarow, Anna-Liisa Hänninen, Taina Suntio, and Ricardo Nunes Bastos

Summary

Proper folding, and consequently exit from the endoplasmic reticulum (ER) and secretion of heterologous exocytic proteins in yeast can be rescued by fusing the proteins to certain yeast-derived polypeptides. Biologically active mammalian glycoproteins can be produced in *Saccharomyces cerevisiae* and *Pichia pastoris* by joining them to a fragment of a natural secretory glycoprotein of *S. cerevisiae*, Hsp150Δ. The performance of the Hsp150Δ carrier in both yeasts appears to exceed that of the MFα leader, which is widely used in industrial protein production. Here we describe the use of the Hsp150Δ carrier in *P. pastoris* in both shake flask and fermentor cultivations. As a reporter protein we use the periplasmic disulfide-bonded *Escherichia coli* enzyme β-lactamase.

Key Words: *Saccharomyces cerevisiae*; *Pichia pastoris*; yeast; secretion; glycoproteins; protein production; Hsp150Δ carrier.

1. Introduction

The secretory pathway of yeast cells provides the means to produce into the culture medium foreign proteins, which require disulfide-bonds and/or *N*-glycosylation for activity ***(1)***. However, many mammalian glycoproteins that are transported through the secretory pathway in their authentic host cells fail to fold correctly in the yeast endoplasmic reticulum (ER), leading to retention in the ER and eventually degradation ***(2,3)***. In yeast cells, proper folding, and thereby ER exit, followed by secretion to the exterior of the cell, can be facilitated by fusing the heterologous protein to a yeast-derived carrier polypeptide, like the prepro fragment of the precursor of the α-mating factor (MFα leader) ***(4,5)***. Using periplasmic disulfide-bonded β-lactamase of *Escherichia coli* as a reporter, we describe here the yeast-derived polypeptide Hsp150Δ, which pro-

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

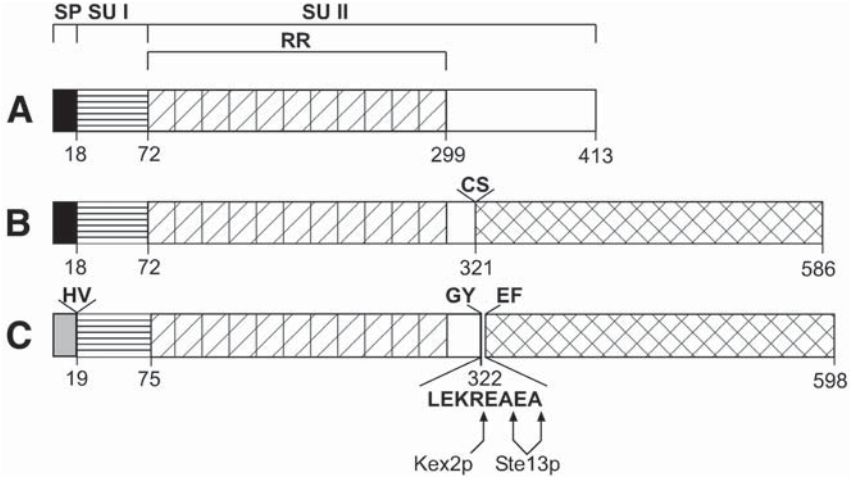

Fig. 1. (A) Schematic presentation of Hsp150. Hsp150 consists of a signal peptide (black box) and subunits I (horizontally striped area) and II. Subunit II is composed of 11 repeats of homologous, mostly 19 amino acid peptides (diagonally striped boxes), and a unique C-terminal fragment (white area). The Hsp150Δ fragment consists of the 321 N-terminal amino acids of Hsp150. **(B)** Hsp150Δ-β-lactamase. The mature portion of β-lactamase is fused to the Hsp150Δ fragment, the 321 first amino acids of Hsp150. **(C)** Hsp150Δ-β-lactamase with Kex2p cleavage site. The chimeric protein consists of the signal peptide of the precursor of MFα (gray box), subunit I and repetitive region of Hsp150, plus the indicated peptide containing a cleavage site for Kex2p and two sites for Ste13p (golgi proteases), followed by the mature portion of β-lactamase. Numbers, last amino acid of each domain; letters, extra amino acids resulting from cloning strategy.

motes proper folding and ER exit of several foreign proteins in *Saccharomyces cerevisiae* and *Pichia pastoris **(6)***.

Authentic Hsp150 (**Fig. 1A**) mostly is secreted to the culture medium ***(7)***. The signal peptide is lost upon translocation into the ER, and the subunits SUI and SUII are detached from each other by Kex2 protease in the late golgi, but remain noncovalently associated to each other. Hsp150 lacks *N*-glycosylation sites, but subunit I and the repetitive region (RR, **Fig. 1A**) are extensively *O*-glycosylated with linear chains up to 5 mannose residues ***(8,9)***. Only the repetitive region of subunit II promotes proper folding of the fusion partner (*see* **Note 1**). Subunit I, however, is necessary because it facilitates translocation of the newly synthesized fusion protein into the ER lumen, at least when the Hsp150 signal peptide is used (*see* **Note 2**).

Depending on the heterologous fusion partner, the Hsp150Δ chimeras are directed to the culture medium ***(10,11)***, or they stay immobilized in the porous wall covering the yeast cells ***(12,13)***. In the latter case, the recombinant yeast cells provide a self-perpetuating source of the heterologous enzyme, which does not have to be purified for use if the substrates and products diffuse freely across the cell wall (*see* **Notes 3** and **4**). The heterologous protein portion is joined to the Hsp150Δ fragment without a cleavage site (**Fig. 1B**). Alternatively, a recognition site is added for the golgi-located Kex2 protease (**Fig. 1C**), which should release the protein product from the carrier polypeptide (*see* **Note 5**).

The *S. cerevisiae* system is well-suited to establish the fate of a foreign protein in the yeast secretory compartment, whereas *P. pastoris* can be used to scale up the production using the methanol-induced strong alcohol oxidase 1 (*AOX*1) gene promoter ***(14,15)***. The promoter is repressed by glycerol, and cultivation in the presence of this carbon source yields high cell densities. After exhaustion of glycerol, methanol is added to induce protein production.

2. Materials

2.1. Bacterial and Yeast Cultures

1. Bacterial strain *E. coli* DH5α (Invitrogen, Carslbad, CA) was cultivated in L-broth (CONDA, Spain) supplemented with 25 µg/mL Zeocin (Invitrogen).
2. *P. pastoris* yeast strain X-33 Mut$^+$ (Invitrogen).
3. Yeast growth media were prepared according to Invitrogen manuals. Yeast extract was purchased from CONDA (Spain), Bactopeptone from OXOID (Hampshire, UK), yeast nitrogen base from DIFCO (Detroit, MI), glycerol from Riedel-de Haen (Seelze, Germany), and methanol from Fluka (Buchs, Switzerland). Zeocin was added to a final concentration of 100 µg/mL (Invitrogen).

2.2. Cloning of Plasmid pKTH4678

1. Vectors pPICZαA and pPICZB from Invitrogen.
2. pKTH4570 or yeast genomic DNA containing ORF YJL159W (*HSP150*).
3. Primer for signal peptide PCR: ACTAG**TTCGAA** ACGATGAGATTTCC hybridizing to nucleotides 938–951 of pPICZαA. Underlined sequence matches template, bold sequence is recognition site for *Bst*BI.
4. Second primer for signal peptide PCR: CATCAGAT**CAGTG**AGCTA ATGCGGAG hybridizing to nucleotides 980–997 of pPICZαA. Underlined sequence matches template, bold sequence is recognition site for *Pml*I.
5. Primers for cloning of *HSP150* fragment ACTAG**CACGTG**GCCTATG CTCCATCTGAGCC and CATCAGAT**GGTACC**CAGAAGTCTTACAGGA GACAGC. Bold sequences are recognition sites for *Pml*I and *Kpn*I, respectively.
6. Primers for cloning of the cDNA of your protein of interest. Design forward and reverse primers containing *Eco*RI and *Xba*I restriction sites, respectively. The

reading frame should be designed according to *Eco*RI site GAATTC, encoding glutamine and phenylalanine.
7. Dynazyme DNA polymerase (Finnzymes, Espoo, Finland).
8. dNTP mix (10 n*M*) (Finnzymes).
9. *Bst*BI, *Kpn*I, *Pml*I, *Eco*RI, *Xba*I, *Xho*I, and *Sac*I restriction enzymes from New England BioLabs (Beverly, MA).
10. T4 DNA ligase from MBI (Fermentas, Lithuania).

2.3. SDS-Polyacrylamide Gel Electrophoresis

1. Separating buffer (8X): 3 *M* Tris-HCl, pH 8.8, 0.1% sodium dodecyl sulfate (SDS).
2. Stacking buffer (8X): 1 *M* Tris-HCl, pH 6.8, 0.1% SDS.
3. Acrylamide/*bis* solution 30% / 0.8% and *N*,*N*,*N*',*N*'-Tetramethyl-ethylenediamine (TEMED) from Bio-Rad.
4. Ammonium persulfate from Sigma Aldrich.
5. Running buffer (10X): 250 m*M* Tris/Base, 1.9 *M* glycine (Sigma-Aldrich), 1% (w/v) SDS.
6. Molecular weight marker from Amersham Biosciences (Little Chalfont, UK).
7. Coomassie stain (Bio-Rad).

3. Methods

3.1. Cloning of an Hsp150Δ Fusion Construct With a Kex2p Cleavage Site and Transformation Into P. pastoris

Use EasySelect *P. pastoris* expression system (Invitrogen) to express, under the control of the *AOX*1 promoter, your protein of interest as an Hsp150Δ fusion protein with a Kex2p recognition site between the fusion partners.

1. To construct plasmid pKTH4678 (**Fig. 2**), polymerase chain reaction (PCR)-amplify the sequence encoding the signal peptide of MFα precursor (**Fig. 2**, sequence IIa), and include recognition sites for *Bst*BI/*Pml*I.
2. Insert the fragment after *Bst*BI/*Pml*I digestion into pPICZB vector (Invitrogen) to create an intermediate vector.
3. Insert in-frame a PCR-amplified fragment encoding amino acids 19–321 of Hsp150 (sequence IIb) to the *Pml*I and *Kpn*I sites of the vector (*see* **Note 6**).
4. Clone the cDNA of your protein of interest devoid of its signal sequence (sequence III) to pPICZαA (Invitrogen) as an *Eco*RI/*Xba*I replacement.
5. Linearize with *Xho*I/*Xba*I the intermediate pPICZB-derived vector.
6. Reclone your cDNA as an *Xho*I/*Xba*I fragment from pPICZαA, including the Kex2p recognition-site sequence, in-frame with the *HSP150Δ* sequence to the intermediate vector to complete plasmid pKTH4678.
7. Use Zeocin™ (Invitrogen) to select *E. coli* transformants.
8. Linearize the expression plasmid pKTH4678 by *Sac*I in order to integrate it into the *P. pastoris* genome.

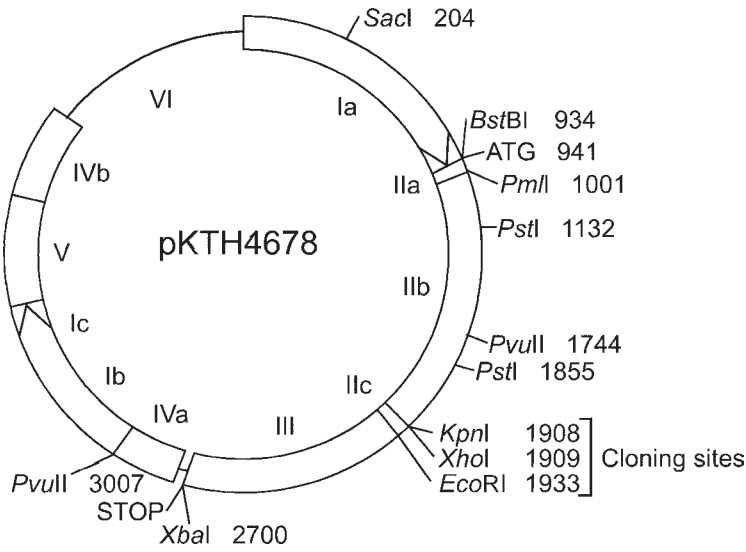

Fig. 2. Graphic map of vector pKTH4678. The sequences of the 5040 base pair plasmid are: Ia, *AOX*1 promoter (nt 1–934); IIa, signal sequence of MFα (nt 941–998); IIb, sequence encoding amino acids 19–321 of Hsp150 shown in **Fig. 1** (nt 1001–1908); IIc, cleavage site for Kex2p (LEKREAEA; nt 1909–1932) (**Fig. 1C**); III, protein product (here β-lactamase, nt 1933–2700); IVa, transcription terminator of *AOX*1 (nt 2788–3129); Ib, TEF1 promoter (nt 3130–3540); Ic, EM7 promoter (nt 3542–3609); V, selection marker for zeocin (nt 3610–3984); IVb, transcription terminator of *CYC1* (nt 3985–4302); VI, contains the bacterial origin of replication. The restriction enzymes and their cleavage sites, which are relevant to cloning, are indicated.

9. Transform plasmid to strain GS115 by electroporation following Invitrogen's instructions.
10. Analyze the yeast Zeocin^R transformants by genomic PCR for presence of the insert.

3.2. Secretion and Release of the Protein Product From the Hsp150Δ Carrier

To describe the protocol, we use here as an example *E. coli* β-lactamase, expressed as an Hsp150Δ-β-lactamase fusion protein with a Kex2p recognition site (**Fig. 1C**).

1. Cultivate recombinant *P. pastoris* strains overnight in shake flasks at 30°C using glycerol as carbon source (BMGY medium, Invitrogen).
2. Dilute in BMMY medium (Invitrogen) to OD_{600} 1, and start adding methanol (0.5% [v/v]) every 24 h, for up to 4 d.

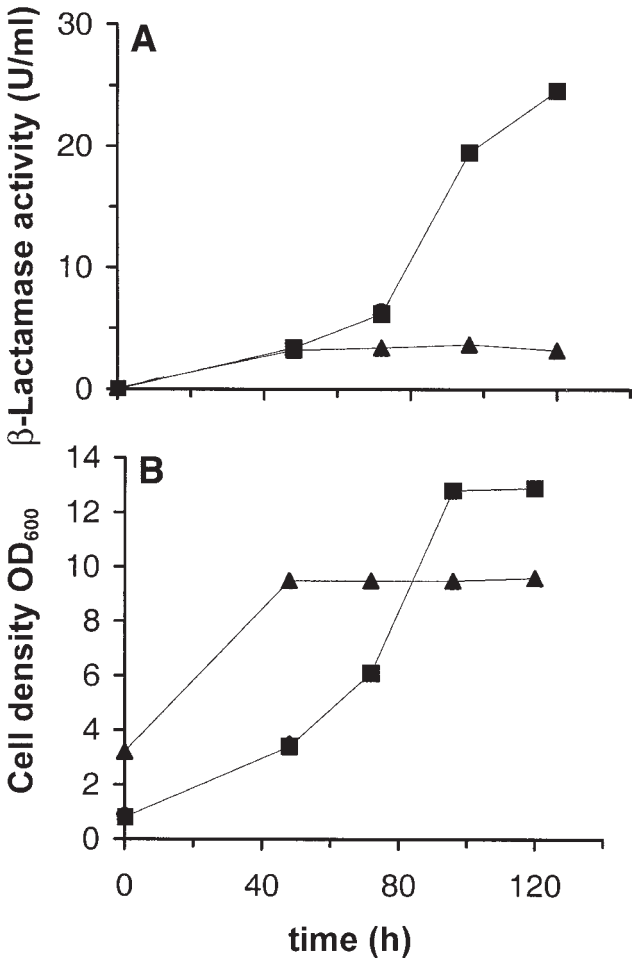

Fig. 3. β-Lactamase activity in the culture medium of *P. pastoris*. Expression of Hsp150Δ-β-lactamase was induced with methanol for 120 h at 30°C in shake flasks and duplicate culture medium samples were assayed for β-lactamase activity (**A**, ■). For reference, the activity secreted to the culture medium of *S. cerevisiae* is shown (**A**, ▲). The squares and triangles in **(B)** show the density of the cell suspensions of *P. pastoris* and *S. cerevisiae*, respectively.

3. Take samples for determination of OD_{600}, biological activity of the protein product (**Fig. 3**) and SDS-PAGE analysis (**Fig. 4**).

3.3. Protein Production in Fermentor Cultivation

Usually much higher yields of the protein product can be obtained in a fermentor by growing the yeast cells to high density using glycerol as the carbon

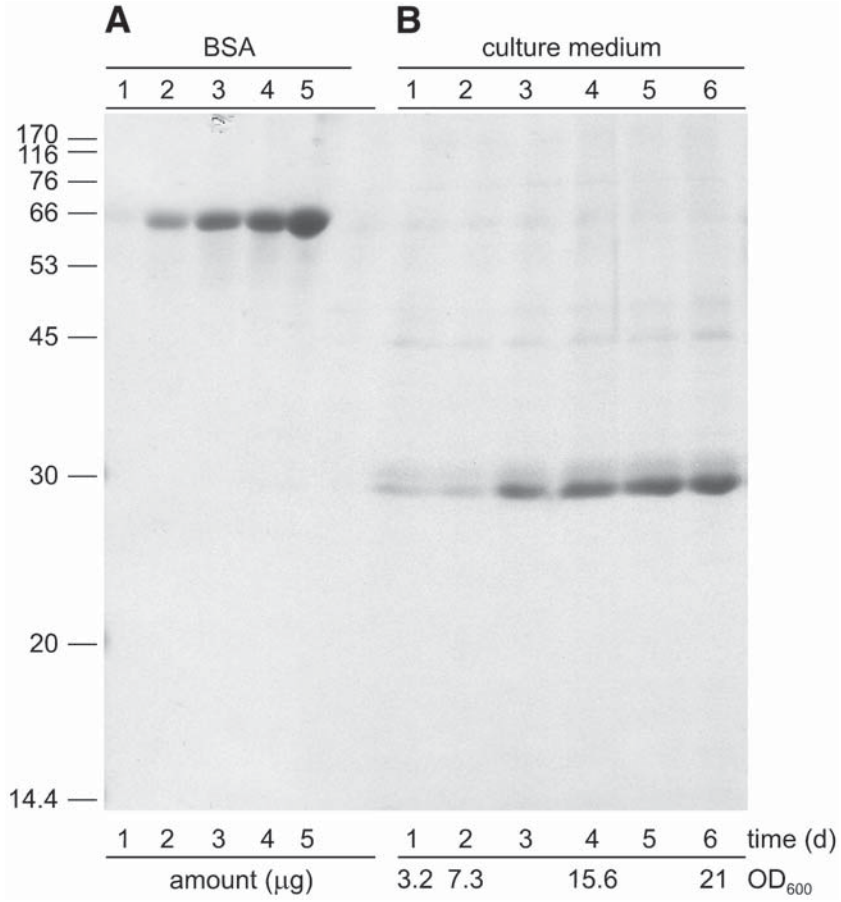

Fig. 4. Release of β-lactamase from the Hsp150Δ carrier. *P. pastoris* was induced to express Hsp150Δ-β-lactamase, as in **Fig. 3**, for the indicated times, and culture medium samples (150 mL) were collected for SDS-PAGE and Coomassie blue staining (**B**, lanes 1–6). β-Lactamase was efficiently released from the Hsp150Δ fragment, because it alone, and no fusion protein, appeared in the medium. Moreover, it was the major protein of the culture medium. Western blot analysis detected almost no intracellular pool of uncleaved fusion protein or released protein product, indicating efficient secretion (data not shown). The indicated amounts of BSA served as reference (**A**, lanes 1–5).

source prior to methanol induction. Methanol is toxic for yeast cells, and in fermentors oxygen can be supplied to accelerate its metabolism. To culture your *P. pastoris* recombinant strain in a fermentor, follow the instructions provided in ***(16)*** and in Invitrogen's *Pichia* fermentation guidelines. When the *P. pastoris* strain used in **Subheading 3.2.** in shake-flask cultivation was fer-

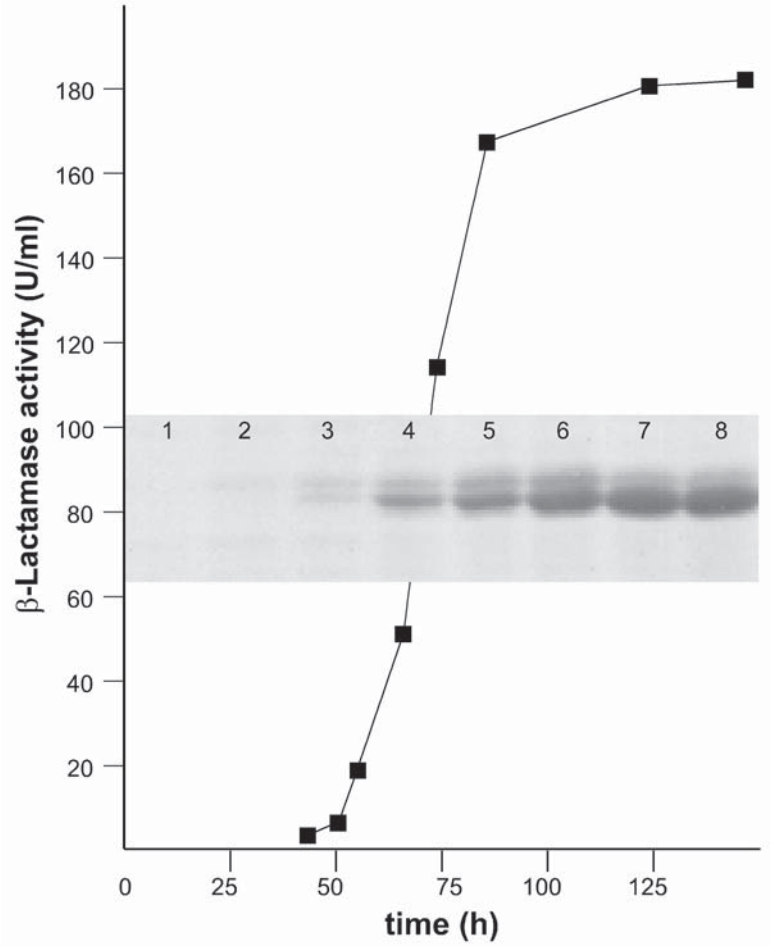

Fig. 5. β-Lactamase production in a fermentor. The recombinant *P. pastoris* strain was grown for 37 h in 2 L of Fermentation Basal Salts Medium, which contained 4% glycerol and was supplemented with PTM1 salts. Thereafter 200 mL of 50% glycerol, supplemented with PTM1 salts, was fed to the culture, until methanol feeding (3.6 mL/h/L) was started at 48 h, when the wet weight of the culture had reached 213 g/L. At 53 h, the flow rate of methanol was doubled. Culture medium samples were measured for β-lactamase activity, which was plotted against time, and 150 µL samples were resolved in SDS-PAGE, followed by Coomassie blue staining of the gel (insert). The lower band (30 kD) is β-lactamase, whereas the upper band may have arisen from incomplete Ste13p processing.

mented in a 4 L fermentor, over seven times more β-lactamase activity was produced into the culture medium, and release of the protein product from the Hsp150Δ fragment was efficient (**Fig. 5**).

4. Notes

1. Why the Hsp150Δ fragment promotes proper folding of heterologous fusion partners in the yeast ER is not known, but we speculate that it has to do with the fact that the fragment does not acquire a regular secondary structure but occurs as a random coil ***(8)***. Thus, it apparently does not impose constraints for the folding of the fusion partner. When attached to subunit I or the C-terminus of entire Hsp150, the foreign fusion protein portion misfolded and remained in the ER, or was deviated from the golgi to the vacuole for degradation, respectively ***(2,10)***. In contrast to the repetitive region, subunit I and the C-terminal fragment do acquire regular secondary structures ***(8,17)***.
2. The Hsp150 signal peptide confers posttranslational translocation into the ER, like signal peptides of many other yeast proteins. They are equally well-suited for protein production as those confering co-translational translocation. However, owing to slow translocation kinetics, in pulse-chase experiments a transient pool of newly synthesized cytosolic Hsp150Δ fusion protein can be detected ***(18)***.
3. The *N*-glycans of recombinant glycoproteins, though produced in Chinese hamster ovary (CHO) cells, are usually undersialylated. This leads to too-rapid clearance of recombinant pharmaceuticals from the blood stream by the hepatic asialoglycoprotein receptor. Recombinant yeast cells expressing α2,3-sialyltransferase activity in the cell wall can be used to increase the sialylation degree of such proteins, simply by incubation of the cells with CMP-sialic acid and the undersialylated glycoprotein ***(13)***.
4. For each foreign protein, the feasibility of the chosen host cell system has to be tested. For instance, though the rat liver α2,3-sialyltransferase ectodomain could be successfully produced to the yeast cell wall as an Hsp150Δ fusion protein ***(12)***, the fate of α2,6-sialyltransferase was different. Unexpectedly, the fusion protein was partly destroyed in the *S. cerevisiae* golgi by GPI-anchored Yps1 protease molecules, *en route* to the plasma membrane. The remaining fusion protein was deviated from the golgi to the vacuole for rapid degradation, though the transferase domain was correctly folded according to kinetic parameters ***(19)***.
5. To achieve efficient cleavage by Kex2p, the recognition site has to be flanked by sites for the Ste13 protease. When the Hsp150Δ fragment was exchanged into the commercially available prepro fragment of MFα precursor, the level of β-lactamase activity and released protein product in the culture medium of *P. pastoris* was about 80% of that obtained with the Hsp150Δ carrier.
6. An extra nucleotide C was created by primer design at the end of the *HSP150* coding region just before the *Kpn*I site, to allow in-frame cloning to the *Xho*I site at the 3' end of the *HSP150* sequence.

Acknowledgments

The financial support of the Academy of Finland (52386, 53607, and 41409), and the National Technology Agency (766/401/97), are acknowledged.

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26

Yeast Two-Hybrid System Screening

R. Daniel Gietz

Summary

The yeast two-hybrid system is a powerful molecular genetic tool conceived by Fields and Song ***(1)***. The article is a comprehensive set of methods designed to take the reader through a yeast two-hybrid analysis of your favorite gene (YFG). This article details the preparation for a screen, the screen itself, as well as the analysis of the positives identified. Using these methods, the readers should be able to successfully negotiate a yeast two-hybrid screen using the Fields' or related systems.

Key Words: *Saccharomyces cerevisiae*; two-hybrid system; protein-protein interaction; GAL4 transcription factor; false positives; cDNA libraries; GAL4 activating domain; GAL4 binding domain.

1. Introduction

The two-hybrid system (THS), first introduced by Fields and Song ***(1)***, is a powerful technique for identifying new proteins involved in specific biological processes. It allows for the rapid isolation of the gene that codes for a protein that interacts with a specific protein of interest.

This article is not a review of the current THS technology, but rather a comprehensive guide designed to take the reader through a THS screen. (If not familiar with the THS, please *see* **refs. *1–5***). Currently, there are a number of different versions of the THS available ***(6)***. This article describes procedures useful for the "Fields" THS. It is based on using either the *Gal4* or *LexA* DNA binding domains and matching yeast strains. Many of the following protocols can be used with other versions with some modification; however, they may not be directly applicable to the Brent Interaction Trap version owing to some distinct differences ***(7,8)***. In addition, this article does not address the use of yeast mating strategy for THS screening. Although these methods are useful for high-throughput screening, it is the opinion of this author that some prey

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

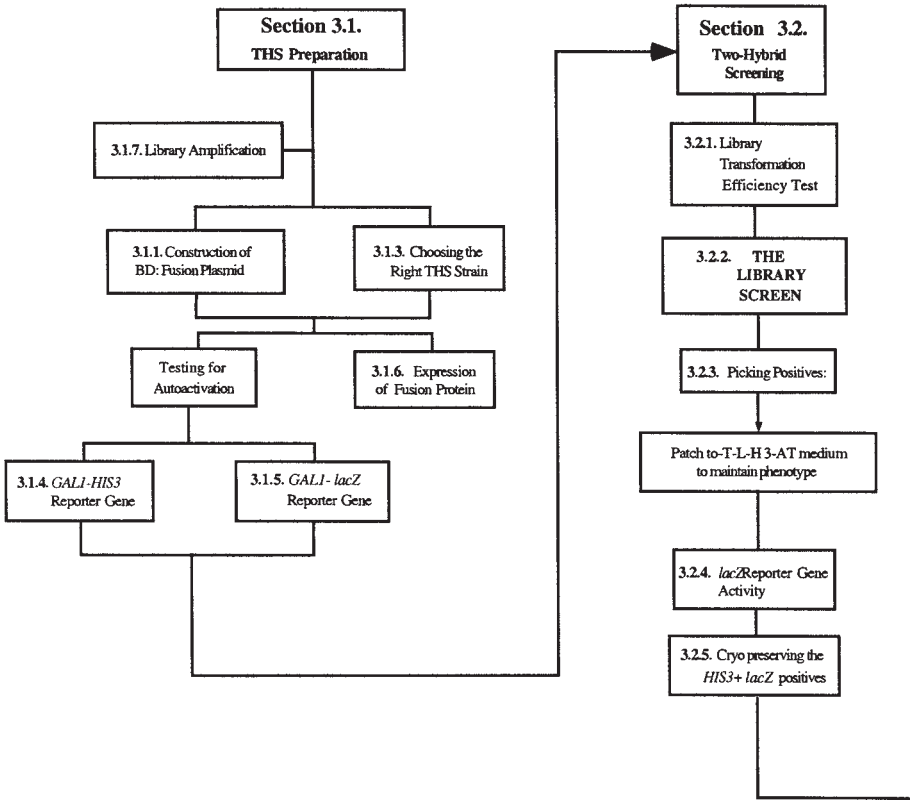

Fig. 1. Two-hybrid system flow chart. The THS flow chart displays the steps required for a THS screen. Begin at **Subheading 3.1.** for preparation of the library, bait gene, and yeast strain. The constructed BD:bait plasmid/yeast strain can then be used in **Subheading 3.2.** to screen a variety of AD:cDNA libraries. **Subheading 3.3.** lists the steps involved in the characterization of putative THS positives. If less than 20 putative positives are obtained from **Subheading 3.3.**, proceed through **Subheadings 3.3.1.–3.3.7.** When more than 20 putative positives are obtained from **Subheading 3.2.**, or when nontypical THS positives are encountered, begin with **Subheading 3.3.10.**, Segregation Analysis, then proceed to analyze true-positives employing the protocols outlined in **Subheadings 3.3.1.–3.3.7.**

plasmids may not be represented in such screens owing to effects on yeast mating. For this reason, the transformation approach will be used here. *See* **ref. *9*** for information about the yeast-mating approach to THS screening.

This article is divided into three parts: **Subheading 3.1.**, Two-Hybrid Screen Preparation describes preparation for a screen; **Subheading 3.2.**, Two-Hybrid

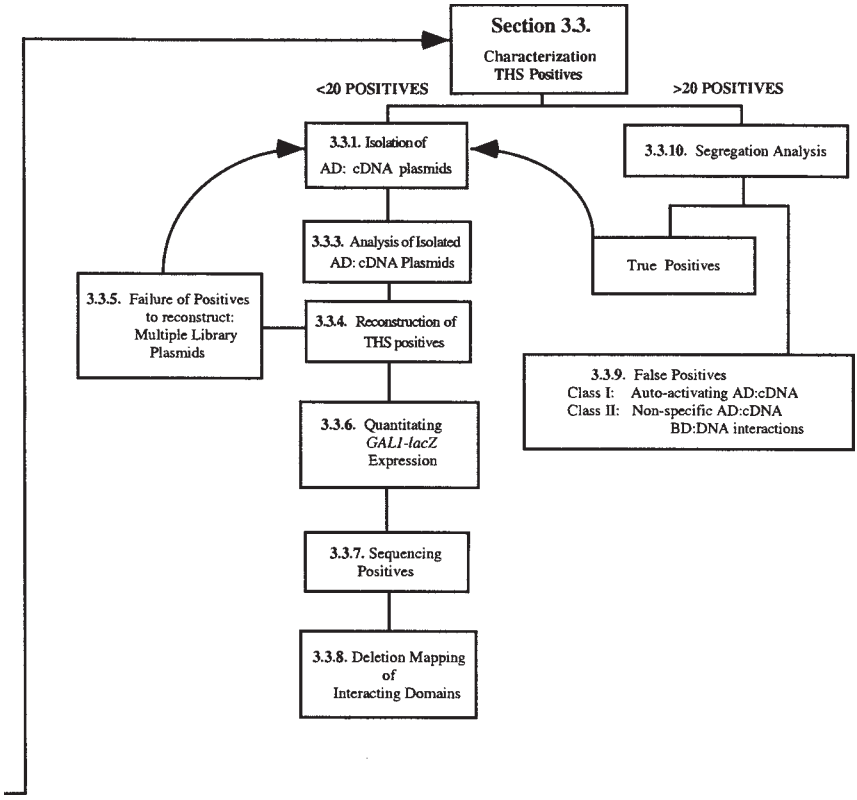

Fig. 1. *(continued)*

Library Screening describes the screen itself; and finally, **Subheading 3.3.**, Characterizing Two-Hybrid Positives describes the analysis of the THS positives. If beginning a two-hybrid screen with an untested bait gene, start with **Subheading 3.1.** to test the bait plasmid and yeast strain combination. This plasmid/strain combination can be used to screen a variety of libraries following the steps outlined in **Subheading 1.5. Figure 1** summarizes the steps and order.

2. Materials

2.1. Testing for GAL1-HIS3 *Auto-Activation*

1. Recipes for all yeast media can be found in ***(10)*** in this volume. Yeast media containing additives such as 3-amino triazole (3-AT) should be produced by adding the appropriate amount of a concentrated filter-sterilized solution after autoclaving and cooling the medium to at least 60°C.

2.2. Testing for Colony β-Galactosidase Activity

1. Z Buffer: $NaH_2PO_4 \cdot H_2O$ 13.79 g/L, KCl 0.75 g/L, $MgSO_4 \cdot 7H_2O$ 0.246 g/L, Titrate with 10 *N* NaOH to pH 7.0.
2. Z buffer/β-ME: This should be made fresh by adding 270 µL of β-mercaptoethanol (β-ME)/100 mL of Z buffer.
3. X-GAL: 20 mg/mL, dissolve 1.0 g of X-GAL in 50 mL of *N*,*N*-dimethylformamide and store at –20°C.
4. Z buffer/β-ME/X-GAL: This should be made fresh by adding 270 µL of β-ME and 1.67 mL of X-GAL solution to 100 mL of Z buffer.

2.3. Preparation of Yeast Lysates for Western Blotting

1. Extraction buffer: 50 m*M* HEPES, pH 7.4, 200 m*M* NaCl. 10 m*M* EDTA (ethylenediaminetetraacetic acid) , 2 m*M* $NaVO_4$, 10 m*M* NaF, 5 µg/mL aprotinin, 5 µg/mL leupeptin, 2 µg/mL E-64 (trans-Epoxysuccinyl-L-leucyl-amido (4-guanidino)butane; *N*-(trans-Epoxysuccinyl-L-leucyl-amido) 4-guanidinobutylamide; L-trans-3-Carbonxyoxiran-2-carbonyl-L-leu-cylagmatine), 2.5 µg/mL pepstatin A, 1 m*M* phenylmethylsulfonyl fluoride (PMSF).
2. Acid-washed glass beads, 425–600 microns (Sigma, cat. no. G-8772).
3. Sodium dodecyl sulfate (SDS) loading buffer: 3% (w/v) SDS, 62.5 m*M* Tris, pH 6.8, 720 m*M* β-mercaptoethanol, 10% (v/v) glycerol, 0.125% (w/v) bromophenol blue.

2.4. AD:cDNA Library Amplification

1. LB (Luria Bertani) Medium + ampicillin (600 mL), tryptone 6 g, yeast extract 3 g, NaCl 6 g, distilled water 600 mL. Titrate to pH 7.0 with 10 *N* NaOH. For plates: add 10 g Difco Bacto Agar to 600 mL volume in each flask prior to sterilization. When cooled to 60°C add 300 µL of a 100 mg/mL stock of ampicillin, mix, and pour plates.
2. Sterile saline: 150 m*M* NaCl, dissolve 8.7 g/L and autoclave.
3. TE (Tris EDTA) Buffer: 10 m*M* Tris, pH 8.0, 1 m*M* EDTA. Make 100 mL by adding 1 mL of 1.0 *M* Tris Cl, pH 8.0, and 0.2 mL of 0.5 *M* EDTA, pH 8.0 to 98.5 mL of double-distilled H_2O.

2.5. Library Transformation Efficiency Test

1. All solutions for transformation can be found in this volume (***10***).

2.6. Isolation of AD:cDNA Plasmid

1. Yeast Lysis buffer, 20 m*M* Tris, pH 8.0, 10 m*M* EDTA, 100 m*M* NaCl, 1% (w/v) SDS, 2% (v/v) Triton X-100. Make 100 mL by adding 2 mL of 1.0 *M* Tris, pH 8.0, 2 mL of 0.5 *M* EDTA, pH 8.0, 2 mL of 5.0 *M* NaCl, 5 mL of 20% (w/v) SDS, and 2 mL of Triton X-100.

2.7. Electroporation of Escherichia coli and Selection of LEU+ Colonies

1. M9 salts (10X): Na_2HPO_4 60 g, KH_2PO_4 30 g, NaCl 5 g, NH_4Cl 10 g, per L of distilled water and autoclave.
2. M9 Leucine prototrophy medium (M9-L): 60 mL 10X M9 salts, 540 mL distilled water, 10 g Difco Bacto-agar. Autoclave this solution and allow to cool to 60°C, then add the following amounts (each solution is sterile) 0.6 mL 1.0 *M* $MgSO_4$, 0.6 mL 0.1 *M* $CaCl_2$ 0.5 mL thiamine (4 mg/mL), glucose (20% [w/v]), 0.15 mL $FeCl_3$ (0.01 *M*), 0.6 mL vitamin B1 (2 mg/mL). Depending on the genetic markers found in your *E. coli* strain, add the appropriate amino acids. For the *E. coli* strain KC8 (genotype; *hsdR*, *leuB600*, *trpC9830*, *pyr::Tn5(kan^r), hisB463*, *lacDX74*, *strA*, *galU*, *galK*) add the following; 6 mL histidine (2 mg/mL), 6 mL uracil (2 mg/mL), 6 mL tryptophan (2 mg/mL).
3. Replicating block (Fisher Scientific, cat. no. 09-718-1; Sigma, cat. no. Z36,339-1) and sterile velveteen.

2.8. Liquid β-Galactosidase Assays

1. *See* **Subheading 2.2.** for buffers.
2. ONPG (*o*-nitrophenyl-β-D-galactopyranoside) 4 mg/mL freshly made in Z buffer.
3. 0.1% SDS (w/v), Dissolve 1.0 g of SDS into 100 mL of distilled water.
4. 1.0 *M* Na_2CO_3, Dissolved 10.59 g in 100 mL of distilled water.

3. Methods

3.1. Two-Hybrid Screen Preparation

3.1.1. Construction of the DNA Binding-Domain Bait Gene Fusion Plasmid

The first step is to construct the DNA binding-domain bait gene fusion plasmid. Your favorite gene (YFG) encoding the protein of interest (considered the bait) is cloned into a suitable THS vector in-frame with the chosen DNA binding domain. There are many different DNA binding-domain vectors available and are listed in ***(6)***. An important consideration is to match the DNA binding-domain plasmid used to the appropriate yeast strain. A few of the DNA binding domain vectors used more frequently are listed in **Table 1** (*see* **Note 1**).

3.1.2. Cloning Strategies

There are a number of different cloning strategies that can be used to clone YFG into a DNA binding domain vector. YFG may be amplified using polymerase chain reaction (PCR) to place the appropriate restriction sites in frame with the multicloning site (MCS) of the DNA binding domain you have chosen. Another strategy we have used is the cloning of YFG into the multicloning site of another vector, such as pUC18, from which the restriction sites can then

Table 1
Two-Hybrid System DNA Binding Domain Plasmid Vectors

Plasmid	DNA binding domain	Restriction sites DNA sequence and frame	Yeast selection marker	Ref.
pAS1	$GAL4_{BD}$	*Nde*I *Sfi*I/*Nco*I *Sma*I *Bam*HI *Sal*I CAT ATG GCC ATG GAG GCC CCG GGG ATC CGT CGA C	*TRP1*	***(11)***
pAS2	$GAL4_{BD}$	*Nde*I *Sfi*I/*Nco*I *Sma*I *Bam*HI *Sal*I *Pst*I CAT ATG GCC ATG GAG GCC CCG GGG ATC CGT CGA CCT GCA GCC	*TRP1* *CYH2*	***(12)***
pGBT9	$GAL4_{BD}$	*Eco*RI *Sma*I *Bam*HI *Sal*I *Pst*I GGA TTC CCG GGG ATC CGT CGA CCT GCA G	*TRP1*	***(13)***
pBTM116	$LexA_{BD}$	*Eco*RI *Sma* I *Bam*HI *Sal*I *Pst*I GGA TTC CCG GGG ATC CGT CGA CCT GCA G	*TRP1*	***(14)***

be used to reclone YFG into frame in one of the DNA binding-domain vectors. Finally, a third strategy we have used often is to blunt-end ligate a restriction fragment containing YFG into the appropriate frame of a DNA binding-domain vector. When cloning any portion of the 5' untranslated region of a gene into a DNA binding-domain vector, ensure that there are no in-frame stop codons. In addition, ensure that cloning your bait gene into the vector does not create an in-frame stop codon, especially if blunt-end ligation was used.

If YFG has identifiable protein domains or motifs, these may be fused to the DNA binding domain for independent study. The most important aspect of this cloning is to ensure that YFG is in-frame with the DNA binding domain so that a fusion protein can be produced (*see* **Note 2**). Any cloning utilizing a PCR strategy should be sequenced owing to the possibility of errors during amplification. In addition the fusion junction of any plasmid constructed using the blunt-end cloning strategy should be sequenced prior to performing any screen to confirm the open reading frame (ORF) fusion. The $GAL4_{BD}$ sequencing primer, 5'TCA TCG GAA GAG AGT AG 3', can be used for the pGBT9, pAS1, and pAS2 vectors. The sequencing primers for pBTM116 are; Fwd 5'GTT GGG GTT ATT CGC AAC 3', Rev 5' CAT AAG AAA TTC GCC CGG 3'.

3.1.3. Choosing the Right THS Reporter Strain

There are many reporter yeast strains now available for the THS analysis ***(6)***. These strains may vary in their reporter constructs and auxotrophic markers, so care must be taken to ensure your vectors and strain match. Yeast strains for the *GAL4* system include reporter constructs that utilize the *HIS3*, *ADE2*, *LEU2*, *URA3*, *CYH2*, *lacZ*, and *MEL1* genes. The *HIS3* reporter allows direct selection of THS positives, however, some strains contain "leaky" derivatives, which require the addition of the chemical 3-amino triazole (3-AT) to the medium to quench background expression of the *HIS3* gene product ***(11)***. The *HIS3* reporter gene also selects for the optimal ratios of fusion proteins to produce reporter gene expression for growth on selective medium ***(15)***. In some strains, the *ADE2* reporter can be used to select for co-activation giving rise to a more stringent screen. The *lacZ* and the *MEL1* reporters can be used to verify positives through co-expression as well as generate quantitative measurements of gene expression.

Another important quality of a THS yeast strain is its transformation characteristics. The ability to generate large numbers of transformants using current transformation protocols is essential for THS screening ***(10)***. The strains listed in **Table 2** all have relatively good transformation characteristics. In addition, both PJ69-4A and KGY37 contain reporter genes that are integrated into their genomes ensuring their maintenance in the absence of selection.

Table 2
Two-Hybrid Yeast Strains

Yeast strain	Genotype	Reporter genes	Plasmid selection	Ref.
Y190	*MAT**a**, ade2-101, gal4 , gal80 , his3 -200, leu2-3,112 trp1 -901, ura3-52, URA3::GAL1-lacZ, lys2::GAL1-HIS3, cyhr^s*	*lacZ HIS3 MEL1*	*TRP1 LEU2 LYS2*	***(11)***
PJ69-4A	*MAT**a**, ade2 , gal4 , gal80 , his3 -200, leu2-3,112 trp1 -901, ura3-52, met1::GAL7-lacZ, ade2::GAL2-ADE2, lys2::GAL1-HIS3*	*lacZ ADE2 HIS3 MEL1*	*TRP1 LEU2 URA3 LYS2*	***(16)***
L40	*MAT**a**, ade2, his3, leu2, trp1, URA3::lexAop(8x)-lacZ, LYS2:: lexAop(4x)-HIS3*	*HIS3 lacZ*	*TRP1 LEU2 ADE2*	***(17)***
KGY37	*MAT**a**, ade2-101, gal4 , gal80 , his3 -200, leu2 -inv pUC18, trp1 -901, ura3 -inv::GAL1-lacZ, lys2 -inv::GAL1-HIS3*	*lacZ HIS3*	*TRP1 LEU2 URA3 LYS2*	***(18)***

3.1.4. Testing for GAL1-HIS3 Auto-Activation

It is important to test each BD:bait plasmid construct for auto-activation of all reporter genes prior to any screen. The activation of reporter genes by the BD:bait plasmid in the absence of an activation domain plasmid is defined as auto-activation. Transform the BD:bait plasmid into the appropriate reporter yeast strain using the rapid transformation protocol ***(10)*** then proceed to test both reporter genes for auto-activation as outlined below.

To test for auto-activation of the *GAL1-HIS3* reporter, yeast cells containing the BD:bait plasmid should be plated, not streaked, onto SC-H (synthetic complete medium minus histidine) medium containing increasing concentrations of 3-AT (1, 5, 10, 25, 50 m*M*). As well, these yeast cells should be plated onto SC-W (synthetic complete medium minus tryptophan) medium to select for the BD:bait plasmid as a control of growth. The addition of 3-AT into the medium is used to suppress the "leaky" nature of this reporter in most strains. The concentration of 3-AT needed to eliminate background growth is plasmid- and strain-dependent. We have found that when screening pAS1 or pAS2 BD:bait plasmid constructs, or when using the strain Y190, higher levels of 3-AT may be required to suppress background *GAL1-HIS3* expression (*see* **Subheading 2.1.**).

1. Grow your yeast transformant containing the verified BD:bait plasmid overnight in SC-W medium to select for maintenance of this plasmid. Alternatively, a 10-µL blob of cells can be scraped from a freshly grown SC-W plate and resuspended in 1 mL of sterile water.
2. Titer the liquid culture using a spectrophotometer (OD_{600} 0.1 = ~ 1×10^6 cells/mL) or a hemocytometer.
3. Plate at least 500 cells/plate onto a pair of SC-W plates as well as pairs of SC-H plates containing 0, 1, 5, 10, 25, and 50 m*M* 3-AT.
4. Incubate at 30°C for up to 5 d.

Examine the SC-H + 3-AT plates for growth. Most BD:bait plasmids will not produce colonies on the medium once the appropriate concentration of 3-AT is reached. The SC-W control plates should contain approx 500 colonies. If you cannot inhibit all growth on SC-H +3-AT even at a 50 m*M* concentration, consider either cloning a different gene fragment into your BD:bait plasmid, or cloning YFG into another BD vector. Another option reduces auto-activation by "dampening" ***(19)*** (*see* **Note 3**).

3.1.5. Testing for Colony β-Galactosidase Activity

In addition to testing for *GAL1-HIS3* auto-activation, it is prudent to also test for *GAL1-lacZ* auto-activation in those strains that contain this reporter gene. This can be accomplished following the protocol listed here. You can use

the pair of SC-W plates that were plated for the *GAL1-HIS3* auto-activation test above (*see* **Subheading 2.2.**).

1. Carefully place a sterile 75-mm circle of Whatman #1 filter paper on top of the colonies or patches growing on selective medium. Ensure that the filter paper makes good contact with the colonies. Mark the orientation of the filter paper relative to the plate using an 18-gauge needle to punch through the filter in an asymmetric pattern.
2. Remove the filter from the plate with sterile forceps after it has become fully absorbed to the colonies and immerse into liquid nitrogen for 10–15 s.
3. Carefully remove the filter from the liquid nitrogen and thaw by placing on a piece of plastic wrap colony-side up. Repeat the freeze-thaw cycle twice more.
4. Place another 75-mm sterile Whatman no. 1 filter into an empty petri plate (100 × 15 mm) and dispense 1.25 mL of Z buffer/β-ME/X-GAL onto the filter.
5. Place the filter, colony-side up, onto a filter paper soaked with Z buffer/β-ME/X-GAL taking care that the filters line up to distribute the solution evenly.
6. Place the lid on each plate and transfer to a plastic bag and incubate at 37°C.

Strong activation of the *lacZ* gene will give a blue color within 1–2 h. If color does not develop, continue to incubate the filters overnight. Note the time needed for color production. A faint blue color after overnight incubation is considered minimal *lacZ* activation.

3.1.6. Preparation of Yeast Lysates for Western Blotting

Prior to screening, we recommend that the steady-state expression of the BD:bait fusion protein be assayed by Western blotting. This may be accomplished if the appropriate reagents are available, such as a specific antibody for the product of YFG. Some vectors, such as pAS1 and pAS2, contain the HA tag ***(15)*** that can be recognized by the commercially available 12CA5 monoclonal antibody (MAb) (Roche Diagnostic Corp.). The $Gal4_{BD}$ antibody (Santa Cruz Biotechnology Inc., or Invitrogen) can also be used. In addition to indicating the expression levels of the fusion protein, Western blotting can verify the in-frame cloning strategy, because the size of the fusion protein detected should compare to the predicted value. Yeast lysates are prepared for electrophoresis following a modified method of ***(20)***.

1. Inoculate the yeast strain containing the BD:bait plasmid into 50 mL of SC-W medium. Incubate at 30°C with shaking until a titer of 1.0×10^7 cells/mL is reached. This may take 16–24 h. Alternatively, a 10-mL overnight culture can be used to inoculate 50 mL to 2.5×10^6 cells/mL and incubate until a titer of $1–2 \times 10^7$ cells/mL is reached. This will take 4 to 6 h or longer in SC-W medium.
2. Collect the yeast cells by centrifugation at 5000*g* for 5 min and wash the cells with 1/2 volume of sterile water. Determine the volume of the cell pellet by adding a specific volume of water and then measuring the total volume of the cell slurry. Transfer the slurry to a 1.5-mL microfuge tube.

3. Resuspend the cells in 2 volumes of ice-cold Extraction buffer.
4. Add 1 volume of glass beads and place each sample onto ice.
5. Vigorously vortex each sample for 30 s and return to ice to cool. Repeat six times for each sample.
6. Centrifuge samples at 14,000*g* at 4°C for 1 min to pellet unbroken cells and cell debris.
7. Transfer the supernatant to another 1.5-mL microcentrifuge tube and cool each sample in an ice slurry for 1 min.
8. Centrifuge each sample again at 14,000*g* at 4°C for 1 min to further clarify extract.
9. Carefully remove supernatant, mix 1:1 with SDS loading buffer, and heat in a boiling water bath for 2 min. These extracts can be used for Western blot analysis and stored at –70°C until needed.

3.1.7. Amplifying the AD:cDNA Library

A library screen can use up to 300 µg of plasmid DNA depending on the yeast strain and BD:bait plasmid. Thus it may be necessary to transform and/or amplify your AD:cDNA plasmid library. A list of libraries can be found in ***(14)***; however, many AD:cDNA libraries are currently commercially available from companies which include; BD Biosciences ClonTech (http://www.bdbiosciences.com/clontech/), Invitrogen (http://www.invitrogen.com). To best amplify all clones within a library, transformed bacterial cells are grown on plates to allow individual colonies to form. We typically plate about 10 times the library complexity for amplification. Most AD:cDNA libraries have complexities of $1–2 \times 10^6$ independent clones; therefore, for good library coverage 10–20 million colonies should be amplified.

Prepare 100 large (150-mm) Petri plates containing the appropriate medium (LB + ampicillin 50 mg/mL depending on your library vector). Also prepare 500 mL sterile saline to be used to resuspend the bacterial colonies. Library plasmid DNA should be transformed into a suitable *E. coli* strain.

1. Titer the *E. coli* library culture by plating 2, 20, and 200 µL of a 10^{-2} dilution onto duplicate LB + Amp (50 µg/mL) plates (100 mm) and incubate overnight at 37°C. Store remaining culture at 4°C.
2. Plate 2×10^5 cells of library culture onto each large (150-mm) LB + Amp plate and incubate until the colonies are fully formed (usually 16–24 h).
3. Harvest the bacterial colonies by flooding a plate with 10 mL of sterile saline and scraping the colonies from the agar surface using a rubber policeman or bent glass rod. Use care not to damage the agar while harvesting bacteria. When complete, dump the liquid onto another plate and repeat. Each 10-mL aliquot can be used for up to five plates. After the solution is saturated with bacteria, transfer to a centrifuge tube. Start the next set of plates with a fresh 10 mL of sterile saline. Repeat until all the plates have been scraped.
4. Mix all aliquots of bacteria together then distribute into 2–250 mL centrifuge tubes. Collect the cells by centrifugation at 10,000*g* for 10 min.
5. Proceed to plasmid DNA extraction ***(21)***.

This procedure will give good amplification of your AD:cDNA library. There is no need to purify the plasmid DNA from the endogenous RNA, because it does not affect yeast transformation (Gietz lab, unpublished results). You are now ready to begin your screen.

3.2. Two-Hybrid Library Screening

This section describes the screening portion of the THS that is carried out after the preparation in **Subheading 1.2.**

3.2.1. Library Transformation Efficiency Test

The first thing that should be done before embarking on a large-scale screen is to perform an AD:cDNA plasmid library transformation efficiency test. This is accomplished by transforming increasing amounts of library plasmid DNA into the two-hybrid yeast strain containing the BD:bait plasmid at a 1X transformation scale. This experiment will allow you to use the library plasmid DNA efficiently, as well as target a specific number of transformants for THS screening. If the DNA concentration used for the transformation is too high, multiple AD:cDNA library plasmids will be transformed into a single yeast cell, making subsequent analysis of two-hybrid positives more difficult.

1. Using the "high-efficiency transformation protocol" ***(10)***, transform increasing amounts of the AD:cDNA library plasmid DNA into your THS yeast strain containing the BD:bait plasmid at the 1X transformation scale (e.g., 0.1 µg, 1 µg, 2 µg , 5 µg, and 10 µg of AD:cDNA library plasmid DNA). Plasmid DNA preparations containing RNA can be estimated for concentration from agarose gels. Incubate the plates for 3–4 d at 30°C.
2. Count the colonies on each set of plates to determine the Transformation Yield (total number of transformants) as well as the Transformation Efficiency (transformants/µg) for each transformation (*see* **Note 4**).

3.2.2. The Library Screen

Once the tranformation yield test has been completed, a large-scale library screen can be performed as outlined in ***(10)*** in this volume. Typically a 30X or 60X transformation scale-up is used. However, the protocol has been successfully scaled up to 120X. Because plating density negatively affects transformation (R. D. Gietz, unpublished), we recommend using at least 50–100 large (150 × 15 mm) Petri plates containing SC-W-L-H + 3-AT medium. Freshly made plates should be allowed to dry for a few days at room temperature to eliminate excessive condensation. Media should also be stored in the dark to prevent a reduction in plating efficiency owing to exposure to fluorescent lighting. Using the appropriate amount of AD:cDNA library plasmid DNA, transform the THS yeast strain containing the BD:bait plasmid using the methods

outlined in ***(10)*** and plate onto medium that selects for reporter gene activation. Plating a 30X or 60X transformation onto 100 large plates can take up to 30 min. Spread the plates out on a counter top, 10 at a time, and dispense 400 µL of transformed cells onto each plate. Using a sterile glass spreading wand, start from the first plate and move to the last, carefully spreading the inoculum onto the surface of the entire plate. Incubate plates in loosely taped Petri plate bags to reduce drying during growth for 4–21 d at 30°C.

3.2.3. Picking THS Positives

Transformation plates should be checked for colonies after 4 d of incubation at 30°C. Continue to check the plates and pick positives every day for the first week and then every 2 d for up to 3 wk. When colonies become visible they should be patched to fresh selection plates (SC-W-L-H + 3-AT) in a grid pattern. These patched plates should be incubated at 30°C until sufficient growth occurs. Colonies that do not produce growth on the patched plate after 5–7 d can be eliminated. When picking positives, be sure to select large colonies that are actively growing. To be certain, observe the colony growth over a number of days. Depending on the strain and BD:bait plasmid, small colonies can usually be found in areas of the plate containing heavy inoculum. Avoid these type of colonies because they are usually not true positives. Positives should be kept on medium that selects for reporter gene activation and all plasmids at all times (e.g., SC-W-L-H + 3-AT plates). This ensures that the BD:bait and AD:cDNA library plasmids encoding the interacting fusion protein are maintained. In cases where a yeast transformant contains multiple AD:cDNA library plasmids, this will ensure the maintenance of the correct plasmid. Yeast colonies maintained on medium containing 3-AT have a reduced viability. Streak or patch to fresh plates weekly and/or cryo-preserve your positives as soon as possible (*see* **Note 5**).

3.2.4. lacZ Reporter Gene Activity

A good indication of a true THS positive is co-activation of all reporter genes. The *lacZ* reporter can be used for this purpose. Once positives are patched and replicated, *lacZ* gene activation can be assayed. It is important to maintain positives on medium that selects for *GAL1-HIS3* reporter activation. This will optimize the expression of fusion proteins to give good levels of reporter gene activity ***(13)***. Assay for colony *lacZ* reporter activity using the method in **Subheading 3.1.5.**

3.2.5. Cryo-Preserving the His+ lacZ+ Positives

Patched colonies that activate the *lacZ* reporter should be cryo-preserved. Streak the *His+ lacZ+* positives onto fresh SC-W-L-H + 3-AT plates and incu-

bate at 30°C for 24 to 48 h. Scrape a blob of fresh inoculum using an inoculating loop or a sterile toothpick and resuspend in 1 mL of sterile 20% glycerol in a 1.5-mL microcentrifuge tube or cryo-tube. Store at –70°C. Alternatively, large numbers of positives can be patched in a grid pattern onto 150-mm SC-W-L-H + 3-AT plates and cryo-preserved using a 96-well microtiter plate replicator (Fisher Scientific). To mark the patching grid onto a plate, carefully place a flame-sterilized replicator onto a fresh plate. This will leave an impression of each prong for patching. After growth, the replicator is sterilized with ethanol and flame and cooled. It is placed onto the grid of patched colonies to make contact with the inoculum. The cells are scraped from the plate by pulling the teeth of the replicator along the surface of the agar without breaking into it. This can be repeated until sufficient inoculum is deposited onto each tooth. Care must be taken not to cross-contaminate different patches. The teeth of the replicator containing the inoculum are then carefully lowered into a sterile microtiter plate (Fisher Scientific) containing 150 µL of sterile 20% glycerol in each well. The cells are washed from the replicator teeth using a gentle rotating mixing action. After the inoculum has been resuspended, the lid is replaced and the plate sealed in a plastic bag and stored at –70°C. Cryo-preserve all positives that activate both reporter genes.

3.3. Characterizing Two-Hybrid Positives

Primary THS positives that activate both the *HIS3* and the *lacZ* reporter genes can now be subjected to further analysis. Owing to the in vivo nature of this system, unforeseen obstacles may be encountered that require you to return to a previous step. If less than 20 positives are obtained, follow **Subheading 3.3.1.** through each step until all are characterized. If greater than 20 positives are identified, proceed to **Subheading 3.3.10.** and use segregation analysis to eliminate false-positives. Continue the analysis with remaining positives by returning to **Subheading 3.3.1.**

3.3.1. Isolation of AD:cDNA Plasmid

To isolate the AD:cDNA library plasmid, nucleic acids are extracted from the yeast cells of each THS positive. A quick and effective method described in ***(22)*** uses glass beads and phenol:chloroform to extract nucleic acids. Alternatively, a method ***(23)*** that uses lyticase to produce spheroplasts can also be used. These nucleic acid preparations will include both *TRP1* and *LEU2* plasmids and should be transformed into an *E. coli* host containing a *leuB* mutation to specifically select for the yeast *LEU2* gene harbored on the AD:cDNA library plasmid.

This protocol, modified from ***(22)***, can be used to isolate DNA from yeast cells grown in either liquid culture or harvested from a plate.

1. Inoculate individual THS positives from SC-W-L-H + 3-AT plates into 2 mL of SC-H or SC-W-L medium and incubate at 30°C overnight. Alternatively, scrape a 50-µL blob of cells from an SC-W-L-H + 3AT plate and resuspend in 500 µL of sterile water in a 1.5-mL microcentrifuge tube.
2. Collect the yeast cells from the liquid culture by centrifugation at 13,000*g* for 30 s.
3. Remove the supernatant and add 200 µL of yeast lysis buffer and gently resuspend the cell pellet using a micropipet tip to avoid the generation of bubbles.
4. Add an approx 200 µL volume of glass beads and 200 µL of buffer-saturated phenol:chloroform (1:1 [v/v]).
5. Vortex each sample vigorously for 30 s and then place on ice. Repeat twice, leaving samples 30 s on ice between treatments.
6. Centrifuge tubes at 13,000*g* for 1 min.
7. Remove the aqueous phase (~200 µL) to a fresh tube and precipitate the nucleic acids by adding 20 µL of 3.0 *M* sodium acetate, pH 6.0, and 500 µL of 95% ethanol. Incubate at –20°C for 30 min and collect the precipitate by centrifugation at 13,000*g* for 5 min at 4°C. Wash the pellet with 100 µL of 70% ethanol (room temp) and dry the pellet for 5 min at room temperature.
8. Dissolve the pellet in 25 µL of TE buffer and store at –20°C.

3.3.2. Electroporation of E. coli and Selection of LEU+ Colonies

The most effective method of transforming a yeast DNA extract into *E. coli* is the electroporation method ***(24)***. The protocol listed here gives electroporation conditions that work with *E. coli* strain DH5α in our hands; however, one should determine the conditions for your strain experimentally. Alternatively, the chemical treatment heat-shock method ***(25)*** can be used to transform *E. coli* (*see* **Subheading 2.6.**).

1. Mix a 2-µL aliquot of extracted yeast DNA with a 25-µL aliquot of electrocompetent KC8, or other *leuB*-containing *E. coli* strain such as MG7α ***(26)*** and place carefully into an cold electroporation cuvet. Keep loaded cuvet on ice.
2. Place electroporation cuvet into electroporation device and pulse the DNA bacterial mixture with the following settings; 25 µF, 1.25 kV, with a pulse controller in parallel with the samples set at 400 .
3. Immediately after pulse, add 1 mL of warm SOC medium to the electroporation cuvet and resuspend the cells. Transfer to a sterile tube and incubate at 37°C for up to 30 min.
4. Plate samples of 25–100 µL onto 2–4 LB + Amp (50 µg/mL) plates and incubate at least 16 h at 37°C. Plating onto LB + Amp medium followed by replica plating onto M9-L plates will save 2 d over plating directly onto M9-L media.
5. The Ap^R colonies are then replica plated, using a replicating block covered with sterile velveteen, onto M9 minimal media minus leucine and incubated for another 16 h at 37°C.
6. Inoculate 4–5 Leu+ colonies per putative positive into 2 mL LB + Amp liquid medium and incubate at 37°C overnight with shaking.

7. Extract the plasmid DNA from these cultures ***(21)*** and dissolve plasmid DNA in 50 µL of TE.

3.3.3. Analysis of Isolated AD:cDNA Plasmids

The *LEU2* AD:cDNA library plasmids isolated from the *leuB E. coli* strain can now be characterized by restriction enzyme digestion and agarose gel electrophoresis. Restriction enzymes that digest on the 5' and 3' ends of the cDNA are vector- and library-specific; check the AD:cDNA library plasmid information. This analysis will group the plasmids by insert size and restriction pattern. Restriction enzyme analysis should be carried out on 4–5 library plasmid isolates from each THS positive. If more than one type of library plasmid is isolated from a single THS positive, further analysis should be carried out with each unique isolate. Independent positives with similar-sized inserts should not be considered duplicates until sequence information can be produced. **Figure 2** shows that with most THS positives each of the 4 plasmid isolates have identical restriction digest patterns, indicating the presence of a single AD:cDNA library plasmid. However, in some cases (lanes B1–B4), the plasmid isolates have different restriction-digest patterns showing the presence of multiple AD:cDNA library plasmids in this THS positive.

3.3.4. Reconstruction of Two-Hybrid System Positives

The next step in the analysis of the THS positives is their reconstruction. Plasmid DNA isolated from the *leuB E. coli* strain is transformed back into the THS yeast strain containing the BD:bait plasmid. Thus, a representative from each plasmid group is tested for activation of both the *HIS3* and *lacZ* reporter genes when in combination with the original BD:bait plasmid. This is accomplished using the high-efficiency transformation protocol ***(10)***. Transformed cells are plated onto SC-W-L as well as SC-W-L-H + 3-AT media. Growth on SC-W-L confirms the presence of both the BD:bait and AD:cDNA library plasmids. Colony formation on SC-W-L-H + 3-AT demonstrates activation of the *HIS3* reporter gene. These His+ colonies can also be tested for activation of *lacZ* reporter using the β-galactosidase assay listed in **Subheading 3.1.5.**

3.3.5. Failure of THS Positives to Reconstruct

The failure to obtain colonies on SC-W-L-H + 3-AT medium while generating colonies on SC-W-L medium suggests that the AD:cDNA library plasmid used in the transformation was not responsible for activation of the reporter genes in the original THS positive. There are two specific situations that are known to give rise to this. The first is the presence of multiple AD:cDNA library plasmids in the original THS positive, caused by transformation with

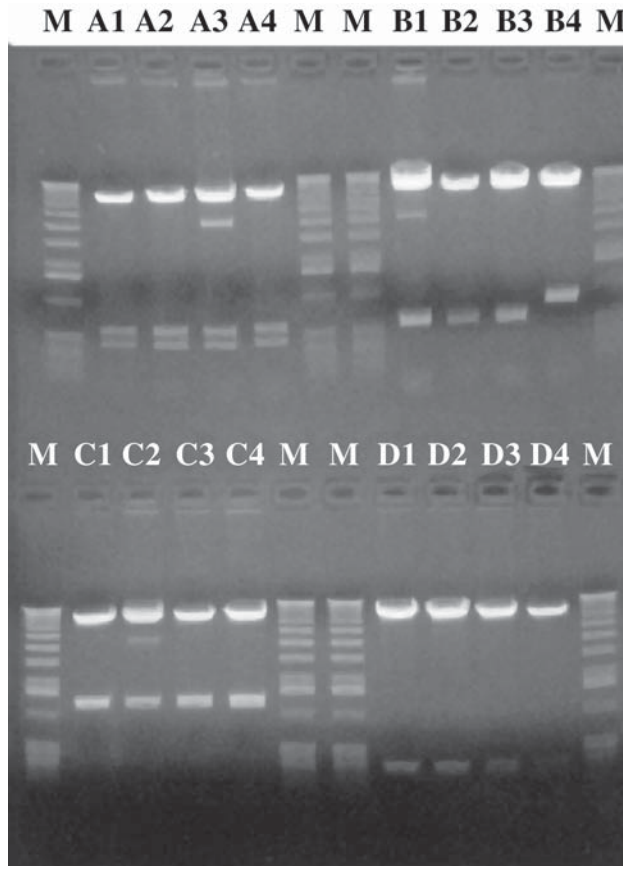

Fig. 2. Plasmids isolated from LEU+ *E. coli*. Nucleic acids were extracted from four independent THS library positives **(A–D)**, and transformed into *E. coli* KC8. Approximately 500 ng of plasmid DNA, from four Leu+ Ap^R isolates (1–4) originating from each positive **(A–D)**, were digested with *Eco*RI, then analyzed on a 0.7% agarose gel. The plasmid isolates are flanked with 1 kilobase (kb) ladder (Lanes M) (Gibco-BRL Life Technologies). Every plasmid isolated from each of the positives A, C, and D (Lanes A1 to A4, C1 to C4, D1 to D4) shows similar restriction pattern and insert size, suggesting the presence of a single library plasmid type within each positive. Conversely, two different restriction patterns are observed in lanes B1 to B4, suggesting the presence of multiple library plasmids in this positive.

high library plasmid DNA concentrations. The second is alteration of the BD:bait plasmid in the yeast strain. Each situation is discussed in **Subheadings 3.3.5.1.** and **3.3.5.2.**

3.3.5.1. Multiple Library AD:cDNA Plasmids

The presence of multiple AD:cDNA library plasmids in a single yeast THS positive is a relatively common occurrence if high plasmid DNA concentrations were used in the library transformation reaction. This situation will be immediately apparent if multiple restriction digestion patterns are identified among the 4–5 AD:cDNA library plasmids originally isolated. Each plasmid type should be tested for reconstruction (*see* **Fig. 2**). If each of the 4–5 AD:cDNA library plasmids have identical restriction patterns and do not reconstruct reporter gene activation, it is likely that your THS positive contains multiple AD:cDNA library plasmids. An additional 10–20 *E. coli* colonies should be isolated from the yeast DNA preparation and analyzed as in **Subheading 3.3.3.** to identify others plasmids that may be responsible for reporter gene activation.

Failure to identify other AD:cDNA library plasmids in a THS positive suggests that it may be nontypical. Depending on the numbers of positives from the screen, these nontypical positives can be retired for later analysis. When time permits, they can be analyzed using the segregation analysis strategy outlined in **Subheading 3.3.10.**

3.3.5.2. Rearranged BD:Bait Plasmids

Some THS false-positives are caused by deletions between direct repeats within the bait cDNA giving rise to an auto-activating BD:bait plasmid ***(27)***. Rare events such as this may be identified using segregation analysis (*see* **Subheading 3.3.10.**).

3.3.6. Quantitating GAL1-lacZ *Expression*

Reconstructed THS positives can be tested for the levels of *GAL1-lacZ* activation using a liquid ONPG assay modified for application to yeast ***(28)***. It is important to assay fresh His+ yeast cultures to ensure that the *lacZ* reporter is induced to optimal levels. Two different protocols are supplied in **Subheadings 3.3.6.1.** and **3.3.6.2.** The SDS-Chloroform method can be used to measure the β-galactosidase activity in positives that turn blue quickly with the filter assay. The liquid nitrogen assay can be used for positives of various strengths and is especially useful when assaying positives that require more than 3 h to turn blue with the filter assay.

3.3.6.1. β-Galactosidase Assay, SDS-Chloroform Method

1. Inoculate individual THS positives from SC-W-L-H + 3-AT plates into 10 mL of SC-H or SC-W-L medium and incubate at 30°C overnight.
2. Titer the overnight culture and subculture into 10 mL fresh SC-H or SC-W-L liquid to a titer of 5×10^6 cells/mL.

3. Incubate with shaking at 30°C until the titer is between 1–2 × 10^7 cells/mL. This should take about 3 to 4 h depending on the positive.
4. Dilute 500 µL of the original culture into 500 µL of water and determine the exact OD_{600}.
5. Aliquot 2 × 1.5 mL from each individual positive into a microcentrifuge tube and pellet the cells at 13,000*g* for 30 s.
6. Remove the supernatant and resuspend each cell pellet in 100 µL of Z buffer.
7. Add 700 µL of Z buffer/β-ME.
8. Add 50 µL of 0.1% SDS and 50 µL of chloroform to each tube and immediately vortex vigorously for 30 s.
9. Add 160 µL of freshly made ONPG (4 mg/mL in Z buffer) and vortex to start the reaction.
10. Incubate at 37°C until a yellow color develops. Incubate reactions for no more than 15 min because little color development will occur after this time. Be sure to stop the reaction when yellow color develops. Incubating reactions too long will reduce unit values owing to saturation of the color development.
11. Stop reactions by the addition of 400 µL of 1.0 *M* Na_2CO_3 and record the elapsed time.
12. Pellet cell debris by centrifugation at 13,000*g* for 5 min. Carefully remove the supernatant and determine the absorbance at 420 nm (A_{420}).
    Calculate units of β-galactosidase activity using the following formula:

$$\text{Units} = (A_{420} \times 1000)/(t \times V \times OD_{600})$$

where t = elapsed time (in min), V = volume of culture used in milliliters, and OD_{600} = optical density of the culture used.

3.3.6.2. β-Galactosidase Assay, Liquid Nitrogen

1. Inoculate individual THS positives from SC-W-L-H + 3-AT plates into 10 mL of SC-H or SC-W-L medium and incubate at 30°C overnight.
2. Titer the overnight culture and inoculate into 10 mL fresh SC-H or SC-W-L liquid to a titer of 5 × 10^6 cells/mL.
3. Incubate with shaking at 30°C until the titer is between 1–2 × 10^7 cells/mL. This should take about 3–4 h depending on the positive.
4. Dilute 500 µL of the original culture into 500 µL of water and determine the exact OD_{600}.
5. Aliquot 2 × 1.5 mL from each individual positive into a microcentrifuge tube and pellet the cells at 13,000*g* for 30 s.
6. Remove the supernatant and resuspend each cell pellet in 100 µL of Z buffer.
7. Pierce the top of each tube with a 26–18-gauge needle before snap freezing tubes. Failure to do so may cause some tubes to **explode,** which can lead to serious injury. Snap-freeze by placing the samples in liquid nitrogen.
8. Thaw the tubes by incubation in a 37°C water bath at least 2 min.
9. Repeat the freeze-thaw cycle two more times.
10. Add 700 µL of Z buffer/BME and vortex.

11. Add 160 µL of freshly made ONPG (4 mg/mL in Z buffer) and vortex to begin reaction.
12. Incubate at 37°C until a yellow color develops.
13. Stop the reaction by the addition of 400 µL of 1.0 *M* Na_2CO_3 and record elapsed time. Stop those reactions that do not turn yellow after 1 h.
14. Pellet the debris by centrifugation at 13,000*g* for 5 min. Carefully remove the supernatant and determine the A_{420}.
    Calculate the units of β-galactosidase activity as described in **Subheading 3.3.6.1.**

3.3.7. Sequencing Positives

Representative members of each group of AD:cDNA library plasmids that reconstruct should be sequenced to identify those positives that contain ORFs in-frame with the $GAL4_{AD}$ Double-stranded plasmid DNA can be sequenced using various commercial kits or companies. The primers used to sequence any *GAL4*-based THS vector can be found in **Table 4**.

DNA sequence information can be analyzed using your favorite DNA analysis software package. Complete or partial THS vector sequence files can be found either at GenBank (http://www.ncbi.nlm.nih.gov/) or the Vector database (http://seq.yeastgenome.org/vectordb/). The sequences from AD:cDNA library plasmids should be analyzed using the BLAST 2.2.9 algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) to identify previously cloned genes in the GenBank database. In addition, the amino acid sequence of the predicted in-frame ORF can be used to search for similarities in a protein database. Positives found to encode short fusion proteins of under 20 amino acids can usually be eliminated from further analysis; however, this decision should be made with reference to additional criterion. For example, the THS was used to successfully identify short peptides that interact with a protein of interest ***(29)***.

3.3.8. Deletion Mapping of Interacting Domains

Further analysis of THS positives included the identification of protein motifs responsible for interaction. Deletions of the bait and library cDNA genes can be generated using restriction sites found within each. It is recommended to begin by deleting the 3' ends of both the bait and library cDNA genes in order to preserve the fusion junctions of the translated proteins. We have found that in some cases, altering the fusion junction can unexpectedly affect the intracellular steady-state level of fusion protein expression.

3.3.9. False Positives

When a large number of positives are recovered from a two-hybrid screen, some are likely to be false-positives. A true THS positive will only produce

Table 4
Sequencing Primers

Primers	Vectors
5'-TCA TCG GAA GAG AGT AG-3'	pGBT9, pAS1, pAS2
5'-TAC CAC TAC AAT GGA TG-3'	pGAD10, pGAD424, pACT, pACT2

Table 5
Two-Hybrid System False-Positives

Class	Behavior	Test
Class I	AD:cDNA library plasmids that do not require the presence of a BD:bait plasmid to activate reporter genes.	Transform AD:cDNA library plasmid into a THS yeast strain alone and test for reporter gene activity.
Class II	AD:cDNA library plasmids that activate reporter genes in the presence of any BD:bait plasmid.	Transform AD:cDNA library plasmid into a THS yeast strain with another unrelated BD:bait plasmid and test for reporter gene activity.

reporter gene activation when in combination with a specific BD:bait plasmid. Listed in **Table 5** are two main classes of false-positives that can occur in a two-hybrid screen.

False-positives can be determined by transformation of the AD:cDNA library plasmid into two different yeast strains: (1) a yeast strain containing **no** plasmid, and (2) a yeast strain containing an **unrelated** BD:bait plasmid. All transformations can be performed when re-constructing your putative positives. Alternatively, co-transformation of each plasmid combination can be performed using the high-efficiency protocol ***(10)***. This allows a single yeast strain to be used for all transformations. Alternatively, segregation analysis (**Subheading 3.3.10.**) can also be useful in discerning class I false-positives as well as identifying THS positives that contain more than one type of AD:cDNA library plasmid. A list of false-positives that have been identified in some two-hybrid screens can be found at the Golemis Lab Home page (http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html).

3.3.10. Segregation Analysis

In those circumstances where there are a large number of THS positives (>20), segregation analysis may be useful to eliminate class I false-positives.

In addition, when it is difficult to isolate the AD:cDNA library plasmid responsible for reporter gene activation, segregation analysis can diagnose this. This technique involves growing the original THS positive yeast strain nonselectively to lose (segregate) the BD:bait and/or the AD:cDNA library plasmid(s) from a portion of the cells. How the loss and/or maintenance of either plasmid affects reporter gene activity is then examined. The phenotype defined by the complement of plasmid(s) within the yeast colony can then be used to identify Type A-E positives (*see* **Fig. 3**).

3.3.11. Segregation Analysis, Nonselective Growth

Nonselective growth of your THS positive can be accomplished in a number of ways. An effective method is to grow the yeast in YPAD medium for at least 10 generations. Alternatively, cells can be grown in SC-L medium to ensure that the AD:cDNA library plasmid is maintained in the yeast cells.

1. Inoculate 5 mL of YPAD or 10 mL of SC-L media to a titer of approx 2×10^4 cells/mL and incubate overnight with shaking at 30°C.
2. Dilute the overnight culture in sterile double-distilled H_2O to give approx 1×10^4 cells/mL, plate 100 µL of this onto each of two SC-L master plates, and incubate 2 d at 30°C. This should give about 1000 colonies per plate.

Fig. 3. *(opposite page)* Segregation analysis of THS positives. Closed and open circles represent colony replicas that did or did not grow, respectively. The positives are classified as either Type A, B, C, D, or E, based on the phenotype that defines the library plasmid. In each case, colony 1 represents the original library positive and contains both the *TRP1* BD:bait and *LEU2* AD:cDNA plasmids and will grow on all types of media. Colonies 2, 3, or 4 display the phenotypes that define them as one of the five types of positives. Type A positives (true-positives) are defined by the phenotype of colony A2. This colony contains only a *LEU2* AD:cDNA library plasmid and fails to activate the *HIS3* reporter gene. This defines the AD:cDNA library plasmid as a true-positive because it requires the BD:bait plasmid to activate the reporter gene. Type B positives (auto-activating positives) are defined by the phenotype of colony B2. This colony contains only a *LEU2* AD:cDNA library plasmid, and activates the *HIS3* reporter gene. This defines the AD:cDNA library plasmid as an auto-activating positive because it does not require the BD:bait plasmid to activate the reporter gene. Type C positives (true-positive with a nonactivating library plasmid) are defined by the phenotype of colonies C2 and C3, respectively. Colony C2 contains both the *TRP1* BD:bait plasmid and *LEU2* AD:cDNA library plasmid and fails to activate the *HIS3* reporter gene. This defines the AD:cDNA library plasmid as a nonactivating library plasmid because it cannot activate the *HIS3* reporter gene even in the presence of the BD:bait plasmid. Colony C3 contains only a *LEU2* AD:cDNA library plasmid and fails to activates the *HIS3* reporter gene. This defines the AD:cDNA library plasmid as an true library positive because it requires the BD:bait plasmid to activate the *HIS3* reporter gene. Type D positives (auto-activating with a nonactivating library plasmid)

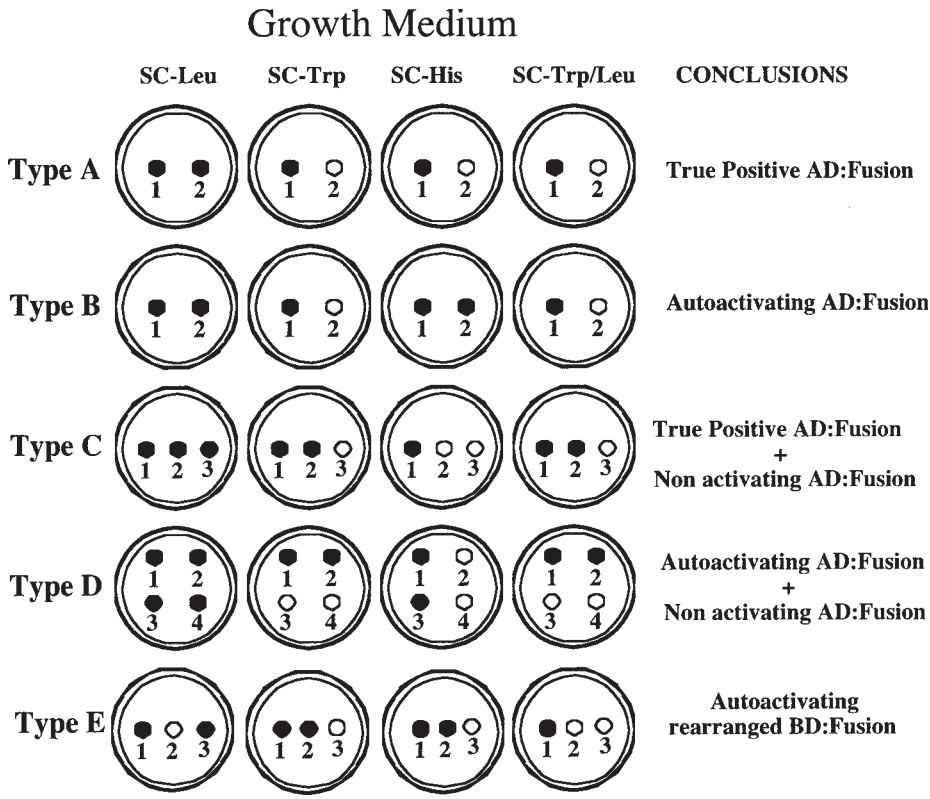

Fig. 3. *(continued)* are defined by the phenotype of colonies D2 and D3, respectively. Colony D2 contains both the *TRP1* BD:bait plasmid and *LEU2* AD:cDNA library plasmid and fails to activate the *HIS3* reporter gene. This defines the AD:cDNA library plasmid as a nonactivating library plasmid because it cannot activate the *HIS3* reporter gene even in the presence of the BD:bait plasmid. Colony D3 contains only a *LEU2* AD:cDNA library plasmid and activates the *HIS3* reporter gene. This defines the AD:cDNA library plasmid as an auto-activating library positive as it does not require the BD:bait plasmid to activate the HIS3 reporter gene. Colony D4 contains only a *LEU2* AD:cDNA library plasmid. The phenotype of D4 is likely the result of the same nonactivating AD:cDNA library plasmid found in colony D2 in the absence of the BD:bait plasmid, and is not a true-positive AD:cDNA library plasmid. This is the result of multiple library plasmids within your library positive. Because colony D3 contains an auto-activating AD:cDNA library plasmid, it is assumed that it is this plasmid that is responsible for reporter gene activation in this positive. Type E positives (auto-activating, re-arranged BD:bait plasmid) are defined by the phenotype of colony E2. This colony contains only a BD:bait plasmid and activates the *HIS3* reporter gene. This defines the BD:bait plasmid as an auto-activating plasmid because it does not require a AD:cDNA library plasmid to activate the reporter gene. Because this plasmid had been previously tested for *HIS3* auto-activation, it is assumed this phenotype is likely the result of a bait plasmid rearrangement. Colony E3 contains only the AD:cDNA *LEU2* library plasmid and only grows on SC-Leu medium.

3. Replica plate the colonies from the SC-L master plates by taking a single impression onto a sterile velveteen replicator and then transfer onto individual plates in the following order; SC-L, SC-W, SC-W-L, and SC-H + 3-AT. Incubate the plates at 30°C for 1–2 d.

Be sure to mark the orientation of each plate before replica plating. Replica plating from a single velveteen impression allows the number of cells deposited onto the SC-H + 3-AT plate to be reduced. This ensures that growth on SC-H + 3-AT plates is owing to reporter gene activation and not heavy inoculum.

There are five different types of growth patterns that will be identified from these plates. These are illustrated in **Fig. 3**. Type A is the pattern seen for true-positives containing a single AD:cDNA library plasmid. Type B occurs for a Class I false-positive, which activates the reporter genes in the absence of a BD:bait plasmid. In this case, no further analysis is required. If a yeast cell containing a true-positive AD:cDNA library plasmid also contains another nonactivating AD:cDNA library plasmid Type C growth pattern will occur. The isolation of the AD:cDNA library plasmid responsible for reporter gene activation in a THS positive containing multiple AD:cDNA library plasmids can be performed from a segregated yeast colony displaying the reporter activation phenotype (*see* **Fig. 3**, Type C colony 1). If your THS positive contains a Class I false positive and another nonactivating plasmid, Type D growth pattern will result. Finally, if your THS positive contains a BD:bait plasmid that has been rearranged, causing it to auto-activate, a Type E growth pattern will be identified. The presence of Type E (*see* **Fig. 3**) positives, BD:bait plasmids that auto-activate can be assayed for using an alteration of the method in **Subheading 3.1.5.** The initial segregation should be done in YPAD (**step 1**) and the master plate must be SC-T (**step 3**).

4. Notes

1. Many vectors have bacterial and yeast marker genes. Most contain a 2-µ yeast replication origin for high copy number in yeast, whereas a few contain ARS CEN sequences giving lower copy number and potentially less toxicity of expressed fusion proteins.
2. We recommend having your cloning strategy checked by a knowledgeable colleague.
3. Auto-activation by the BD:bait construct does not necessarily mean the end of your screen. Cloning your bait gene into a different vector, such as pGBT9, may reduce the auto-activation if pAS1 or pAS2 were used previously. Alternatively, the construct can be modified by deletion to remove the region responsible for the auto-activation.
4. The example in **Table 6** shows that as the DNA concentration increases in the transformation reaction, the transformation yield increases but the transformation efficiency decreases. It is best to scale-up the transformation reaction rather

Table 6
Transformation Efficiency and Yield Example

DNA amount	Colonies/ plate average	Transformation yield (Total Transformants[a])	Transformation efficiency (Transformants/μg[b])
0.1 μg	255	255,000	2.6×10^6/μg
1.0 μg	1545	1,545,000	1.5×10^6/μg
2.0 μg	1765	1,765,000	0.8×10^6/μg
5.0 μg	1894	1,894,000	0.3×10^6/μg
10.0 μg	2019	2,019,000	0.2×10^6/μg
20.0 μg	2208	2,208,000	0.1×10^6/μg

[a]Calculation of transformation yield. Total Transformants = [(colonies/plate)/(volume/plate)] × [(volume in μL of total reaction)/(dilution factor)] e.g., In this example for the 0.1 μg transfromation the colonies/plate were 251 and 259 giving an average of 255. A 10 μL volume of a 10-1 dilution was plated and the final volume of the transformation reaction was 1000 μL. [(255 colonies/plate)/(10 μL/plate)] × [(1000 μL/reaction)/(10^{-1})] = 255,000 colonies/reaction

[b]Calculation of transformation efficiency. Transformants/μg = (Transformation yield)/(amount of DNA in μg)

than increase the amount of DNA transformed to limit the production of transformants that contain multiple library plasmids. From these data the DNA concentration to use for a library screen is either 1 or 2 μg for each 1X scale transformation. Performing a 30X scale-up should produce 46 to 52 million transformants with 30–60 μg of AD:cDNA library plasmid DNA.

5. Be aware that other bacterial and fungal contaminants will likely occur on the screening plates. Use caution when picking from plates containing colonies with a different coloration or texture. Plates heavily contaminated with filamentous fungi-producing conidia should be discarded. In many cases, attempts to rescue colonies from such plates will only further contaminate the laboratory air space.

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Import of Precursor Proteins Into Isolated Yeast Mitochondria

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Summary

Mitochondria fulfill a large variety of metabolic tasks such as respiration, beta-oxidation, heme biosynthesis, ketone-body, or amino acid synthesis. In addition to their metabolic role, mitochondria are also key players in cellular apoptosis and participate in the generation of reactive oxygen species (ROS) and in calcium signaling. The proteins involved in these processes are mostly encoded by nuclear DNA and synthesized on cytosolic ribosomes. Accordingly, they have to be transported into mitochondria in order to reach the place where they act. The process of mitochondrial protein import can be reconstituted in vitro using isolated mitochondria and in vitro synthesized precursor proteins.

Key Words: Mitochondria; protein import; precursor protein; protein translocation; in vitro import.

1. Introduction

The majority of mitochondrial proteins are encoded in the nucleus. They are synthesized on cytosolic ribosomes and must be targeted to and imported into mitochondria. The precursor proteins contain targeting signals that ensure the fidelity of the import process. Most matrix-destined precursor proteins possess an N-terminal presequence, whereas polytopic membrane proteins of the inner and outer mitochondrial membrane usually utilize internal targeting signals. In the presence of ATP, the precursor proteins are released from cytosolic chaperones, which keep them in a loosely folded, import-competent state. They are recognized at the mitochondrial surface by the receptors of the Translocase of the Outer mitochondrial Membane (TOM) and are subsequently transported across the outer membrane by the TOM complex. Proteins destined for the inner membrane or the matrix are only imported if the membrane potential

From: *Methods in Molecular Biology, vol. 313: Yeast Protocols: Second Edition*
Edited by: W. Xiao

($\Delta\psi$) across the inner membrane is present. Using this electrochemical gradient as the initial energy source, the import process is initiated by one of the Translocases of the Inner mitochondrial Membane (TIM complexes). In addition, mitochondrial matrix proteins require the assistance of the matrix Hsp70 (Ssc1) driven by ATP hydrolysis, for completion of their import. In most cases, the N-terminal targeting signal of the precursor protein is cleaved off by the mitochondrial matrix processing peptidase, generating the mature protein ***(1,2)***.

The prerequisites for reconstitution of these processes in in vitro import experiments are the synthesis or isolation of a precursor protein, isolation of mitochondria (*see* Chapter 5), plus a check of the integrity of the mitochondria. The import experiment can generally be divided into the import reaction and the subsequent analysis.

For the in vitro import reaction, it is important to decide if a radiolabeled precursor protein or chemical amounts of a purified precursor protein should be used. Usually, radioactive amounts (as described in this chapter) are more convenient to work with. However, experiments that aim to saturate the import complexes require the use of chemical amounts of precursor protein ***(3)***. In this case, precursor proteins overexpressed in *Escherichia coli* are employed, although proteins isolated from yeast can also be used. The next decision considers the energetic conditions for the import reaction. For a standard import reaction, ATP and NADH are added. In order to generate translocation intermediates, special conditions have to be used. Depending on the purpose, this can be the variation of the external or internal nucleotide levels ***(4)*** of the mitochondria, the reduction or dissipation of the membrane potential ***(5)***, or the use of fusion proteins with tightly folded domains (e.g., dihydrofolate reductase in the presence of its inhibitor methotrexate), which can be arrested in the import channels ***(6)***. Alternatively, mitochondria derived from *Saccharomyces cerevisiae* strains carrying mutations within components of the translocation machinery can be employed. Such strains are usually grown at the permissive temperature in order to maintain the mutated protein functional. Mitochondria are then isolated and the defect can often be induced by a short heat treatment of the isolated mitochondria before the in vitro import assay. Interestingly, mutant phenotypes appear to be more pronounced in the in vitro import studies.

The analysis of the import reaction involves the detection of the imported precursor protein and the determination of its mitochondrial localization. To distinguish between proteins in the matrix or the intermembrane space of mitochondria, it is possible to selectively rupture the outer mitochondrial membrane by osmotic swelling and subsequently treat with protease. This is achieved by incubation of mitochondria in a buffer with low osmolarity (25 m*M* sucrose), which leads to swelling of the matrix and the subsequent rupture of the outer membrane ***(7)***. To discriminate between membrane-integrated,

peripheral, and soluble proteins, mitochondria can be treated with Na_2CO_3 ***(8)*** or salts after the import reaction. With these straightforward assays, most mitochondrial proteins can be classified. The in vitro import system also allows for the monitoring of the assembly of multimeric proteins on native gels ***(9,10)***. This is especially useful for outer membrane proteins, for which it is difficult to differentiate between unspecifically membrane-associated precursor proteins and specifically imported ones. Finally, the radiolabeled precursor protein is detected by autoradiography or digital autoradiography (Phosphor Imager), which allows for quantitative analysis.

2. Materials

Prepare all solutions in distilled H_2O, unless otherwise stated.

1. Transcription buffer: 400 m*M* HEPES-KOH, pH 7.4, 60 m*M* $Mg(OAc)_2$, 20 m*M* Spermidine in RNAse-free H_2O, stored in 400 µL aliquots at –20°C (stable for years).
2. Transcription premix: Mix 400 µL Transcription buffer, 20 µL 20 mg/mL bovine serum albumin (BSA) (for molecular biology, e.g., Roche, Mannheim, Germany), 40 µL 1 *M* dithiothreitol (DTT), 20 µL 0.1 *M* ATP (nucleotides as 0.1 *M* solution, e.g. from Amersham, Uppsala, Sweden), 20 µL 0.1 *M* CTP, 20 µL 0.1 *M* UTP, 2 µL 0.1 *M* GTP (*see* **Note 1**), 2.67 mL RNAse-free H_2O, and store 120-µL aliquots at –80°C (stable for years).
3. RNasin (40 U/µL, e.g., Promega, Mannheim, Germany)
4. 1 m*M* Cap: 1 m*M* diguanosin triphosphate, sodium ($m^7G(5')ppp(5')G$, e.g., Amersham) solution in RNAse-free H_2O, aliquot in 10 µL and freeze at –20°C (stable for years).
5. SP6 or T7 RNA polymerase (50 U/µL, e.g., Stratagene, La Jolla, CA).
6. 10 *M* LiCl in RNAse-free H_2O.
7. Ethanol and 70% ethanol.
8. Rabbit reticulocyte lysate system (Amersham) (*see* **Note 2**).
9. 7.15 µCi/µL ^{35}S-methionine (70%)/cysteine (30%) (e.g., Pro-Mix L-[^{35}S] in vitro, Amersham)
10. 200 m*M* methionine (unlabeled, 1 mL aliquots, store at –20°C, stable for years).
11. 1.5 *M* sucrose (1-mL aliquots, store at –20°C, stable for years).
12. Radioactive ink: Mix 1 mL black ink with 1–2 µL ^{35}S-methionine/cysteine.
13. SEM: 250 m*M* sucrose, 1 m*M* ethylenediaminetetraacetic acid (EDTA), 10 m*M* MOPS-KOH, pH 7.2; EM: 1 m*M* EDTA, 10 m*M* MOPS-KOH, pH 7.2 (store at 4°C, stable for weeks)
14. Isolated mitochondria in SEM 10 mg/mL (protein content) frozen at –80°C (*see* Chapter 5) (stable for years).
15. Prot. K (freshly made): 10 mg/mL or 1 mg/mL Proteinase K in SEM.
16. PMSF (Phenylmethanesulfonyl fluoride, freshly made): 200 m*M* PMSF (handle with care: toxic) in isopropanol (PMSF is more stable in isopropanol than in ethanol). PMSF is unstable in water and must be added just prior to use.

17. For the incubation of the many import samples on ice, we use galvanized aluminium blocks, with holes milled for Eppendorf tubes, placed on ice.
18. Import buffer: 10 m*M* MOPS-KOH, pH 7.2, 250 m*M* sucrose, 80 m*M* KCl (*see* **Note 3**), 5 m*M* $MgCl_2$, 2m*M* KH_2PO_4, 3% BSA (essentially fatty acid-free, e.g., from Sigma, Taufkirchen, Germany), aliquot in 1.25 mL, and freeze at –20°C (stable for years).
19. MOPS/met: 500 m*M* MOPS-KOH, pH 7.2, 100 m*M* methionine (1-mL aliquots, store at –20°C, stable for years).
20. ATP: 200 m*M* pH 7.2 (KOH), freeze in 30-µL aliquots at –20°C (stable for years).
21. NADH (freshly made): 100 m*M* in SEM.
22. 1 m*M* valinomycin (handle with care; toxic; K^+-ionophor, disrupts the membrane potential) in ethanol (store at –20°C, stable for years).
23. 10 m*M* oligomycin (handle with care: toxic; blocks the F_oF_1-ATPase, ATP cannot be used to generate a membrane potential through reverse action of the F_oF_1-ATPase) in ethanol (store at –20°C, stable for years).
24. Mix 100 µL of 1 m*M* valinomycin, 100 µL of 10 m*M* oligomycin with 800 µL ethanol (store at –20°C, stable for years), this mix is later referred to as VO.
25. StrataClean, Stratagene.

3. Methods

3.1. Generation of Radiolabeled Precursor Proteins

1. Clone the continuous (without introns) protein coding sequence of interest into a suitable vector (e.g., pGEM-4Z) in the correct orientation downstream of the Sp6 or T7 promotor (*see* **Note 4**). The correct initiation codon should be relatively close to the promotor and the first AUG in the RNA to be synthesized. Isolate and purify this vector (e.g., Plasmid Maxi Kit, Qiagen, Hilden, Germany) and resuspend it in sterile RNAse-free H_2O. For a screen, a single experiment or for the generation of N- or C-terminal deletions one can alternatively use a PCR product; *see* **Note 5**).
2. Start the in vitro transcription: Combine 120 µL of transcription premix, 5 µL RNasin (40 U/µL), 10 µL 1 m*M* Cap (*see* **Note 1**), 20 µg of plasmid DNA (*see* **Note 6**), 2 µL RNA polymerase (50 U/µL) (either Sp6 or T7 depending on the promotor used); add sterile RNAse-free H_2O to 200 µL mix and incubate at 37°C for 1 h (*see* **Note 7**).
3. To precipitate the RNA, add 20 µL 10 *M* LiCl and 600 µL ethanol, mix, and store at –20°C.
4. After at least 30 min (up to overnight) spin for 30 min at 12,000*g* and 2°C.
5. Remove the supernatant and dry the RNA pellet at room temperature (approx 5–10 min; *see* **Note 8**).
6. Resuspend the mRNA pellet immediately in 100 µL sterile RNAse-free H_2O containing 1 µL RNAsin (40 U/µL). Aliquot into 25 µL samples (*see* **Note 9**) and freeze at –80°C (stable for years).
7. For the in vitro translation, thaw the RNA and the reticulocyte lysate on ice. Mix 70 µL RNAse-free H_2O, 20 µL amino acid mix minus methionine, 10 µL KOAc,

5 µL $Mg(OAc)_2$ (supplied with the reticulocyte lysate system), 20 µL ^{35}S-methionine/cysteine, 25 µL RNA, and 100 µL reticulocyte lysate, and incubate 1–2 h at 30°C (*see* **Note 10**).

8. Add 4 µL 200 m*M* unlabeled methionine (*see* **Note 11**). Spin for 30 min at 125,000*g* and 2°C to sediment ribosomes.
9. Add 27 µL 1.5 *M* sucrose to make the lysate isotonic to the import buffer. The lysate is subsequently aliquoted (e.g., 50 µL) to minimize freeze-thaw cycles of the precursor protein and stored at –80°C (half-life of ^{35}S is 87 d).
10. To check the quality of the lysate add 1 µL to sample buffer, incubate 5 min at 95°C and run an sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
11. Stain, destain and dry the gel. Mark the molecular-weight markers by applying the radioactive ink with a wooden toothpick. Put scotch tape across the dots of radioactive ink, expose the gel to an autoradiography cassette (if the translation standards are always exposed for similar times, e.g., overnight, it is easier to compare the quality of the new lysates with previous ones), and develop using a Phosphor Imager.

3.2. Protein Levels and Integrity of the Outer Mitochondrial Membrane

In many in vitro experiments, mitochondria from different yeast strains have to be compared. To ascertain that these are of similar quality, the yeast cultures should be grown in parallel and under identical growth conditions (respiratory states). In addition, the mitochondria should be isolated in parallel. Prior to any import experiment, the protein levels of the mitochondria used should be compared and the integrity of the outer mitochondrial membrane has to be checked.

1. Thaw 40 µL of mitochondria (10 mg protein/mL) on ice (*see* **Note 12**).
2. Dilute 20 µL mitochondria into 800 µL SEM (ice-cold) and split into 4 × 200 µL.
3. Prepare two fresh Proteinase K stock solutions one of 10 mg/mL and a second of 1 mg/mL (e.g., by diluting the 10 mg/mL stock 1/10 in SEM buffer). Add to the different tubes 2 µL or 10 µL 1 mg/ml Proteinase K, respectively, and 2.5 µL or 5 µL 10 mg/mL Proteinase K, respectively; mix and incubate for 15 min on ice.
4. Add 2 µL 0.2 *M* PMSF to each tube, mix, and incubate for 10 min on ice.
5. Spin (5 min, 12,000*g*, 2°C), carefully remove the supernatant from the mitochondrial pellet (*see* **Note 13**), wash with SEM buffer containing 2 m*M* PMSF (do not resuspend the mitochondrial pellet), and spin (5 min, 12,000*g*, 2°C).
6. Remove the supernatant and add sample buffer to the pellet. In separate tubes, prepare 2.5, 5, or 10 µL mitochondria with sample buffer (as reference amounts), run an SDS-PAGE, perform a Western-transfer, and immunodecorate for selected marker proteins (*see* **Fig. 1**).
7. Control if protease-sensitive intermembrane space proteins have remained inaccessible to the protease. Compare the protein levels of different mitochondrial preparations. If necessary, readjust the ratio of mitochondria from different preparations to have comparable amounts of marker proteins for parallel in vitro import experiments.

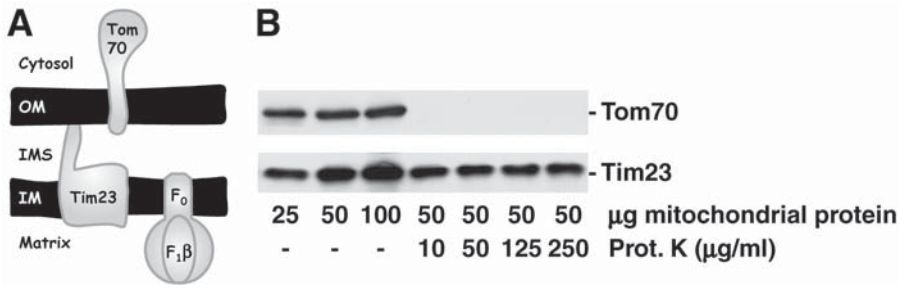

Fig. 1. (A) Topology of selected proteins used as markers for protease treatment and import reactions. Tom70, integral outer membrane protein with large cytosolic domain. Tim23, integral inner membrane protein with a domain protruding into the intermembrane space. $F_1\beta$, matrix protein peripherally associated with the inner membrane. **(B)** Increasing concentration of mitochondria without protease treatment and identical amounts of mitochondria treated with increasing concentrations of Proteinase K (Prot. K) were analyzed by Western blotting for the mitochondrial marker proteins Tom70 and Tim23. The cytosolic domain of Tom70 is degraded at low protease concentrations, whereas Tim23 remains protected against proteolysis by the intact outer mitochondrial membrane.

3.3. Import of Precursor Proteins

A general import reaction for radiolabeled precursor proteins with a presequence is set up as follows: Mix 100 µL of import buffer, 5 µL MOPS/met (*see* **Note 14**), 1 µL ATP, 2 µL NADH, and lastly add 2.5 µL of mitochondria (10 mg protein/mL). To start the import reaction, incubate the import mix for 2 min at 25°C and start by addition of 1–20 µL reticulocyte lysate containing the radiolabeled precursor protein. Mix on a vortex at low speed and incubate for the desired time at 25°C. To terminate the import reaction, add 1 µL of VO and chill the mixture on ice. To remove unspecifically bound precursor from the surface of the mitochondria, protease treatment can be performed (*see* **Fig. 2**). As an example, we will describe an import reaction of Su9-DHFR (presequence of *Neurospora crassa* F_o-ATPase Subunit 9 fused to the passenger protein dihydrofolate reductase). Owing to its good radiolabeling and high import efficiency, Su9-DHFR has proven to be an excellent tool for in vitro imports.

1. Thaw 20 µL mitochondria and 8 µL ATP on ice.
2. Prepare import mix of ice-cold 800 µL import buffer, 40 µL MOPS/met (*see* **Note 14**), 16 µL fresh 0.1 *M* NADH, 8 µL ATP, and 20 µL mitochondria (10 mg protein/mL).
3. Take 200 µL of the mix and add 2 µL VO (*see* **Subheading 2.2., item 4**); add 6 µL ethanol (mock) to the remaining 600 µL mix, mix, and incubate 2 min at 25°C.

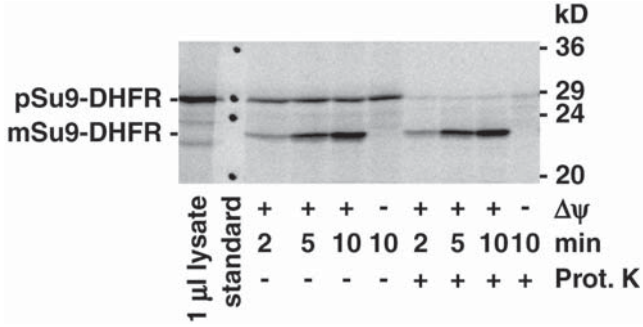

Fig. 2. In vitro import of the precursor protein Su9-DHFR. The precursor (p) is imported into the mitochondrial matrix and processed to the mature (m) form in a time-dependent manner. In the absence of a membrane potential ($\Delta\psi$), import is inhibited. The amount of precursor that is bound unspecifically to mitochondria does not display any increase over time nor dependence on the membrane potential. Unspecifically associated precursor at the outer surface of mitochondria is degraded by externally added Proteinase K (Prot. K).

4. Add 15 µL Su9-DHFR lysate to the 600 µL mix, mix and incubate at 25°C (start).
5. After 1 min add 5 µL Su9-DHFR lysate to the VO-treated 200 µL mix, mix on a vortex at low speed, and incubate at 25°C.
6. Pipet 2 µL VO into three tubes and place them on ice.
7. After 2, 5, and 10 min take a 200-µL sample out of the 600-µL import reaction, and add to the new tubes that contain VO, mix, and leave on ice.
8. After 11 min, move the 200-µL VO pretreated import mix on ice.
9. Split all samples into new tubes (2×95 µL).
10. Add to one-half 2.5 µL 1 mg/mL Proteinase K, mix, and incubate for 15 min on ice.
11. Add 1 µL 0.2 *M* PMSF to all samples, mix, and incubate for 10 min on ice.
12. Spin all samples (5 min, 12,000*g*, 2°C), carefully remove the supernatant (*see* **Note 13**), wash with SEM buffer containing 2 m*M* PMSF (do not resuspend the mitochondrial pellet), and spin again (5 min, 12,000*g*, 2°C).
13. Pipet off the supernatant and add sample buffer. In addition, prepare a sample of 0.5–1 µL reticulocyte lysate (10–20% of the amount used for the import reaction). Run an SDS-PAGE, stain, destain, dry, and expose to a phosphoimager screen. Develop after overnight exposure with a Phosphor Imager (*see* **Fig. 2**).

3.4. Analysis of the Import Reaction

To check if and how much of the imported precursor protein has reached its proper localization, further analysis has to be performed. Compare the import reactions of intact, swollen, and lysed mitochondria with and without protease treatment. With intact mitochondria, the protease treatment will only degrade unspecifically bound precursor proteins on the outer surface and outer mem-

brane proteins, whereas, with swollen mitochondria, proteins with domains in the intermembrane space will be degraded as well. After lysis of the mitochondria with detergent, soluble matrix proteins are released into the supernatant and therefore all of the imported proteins will be degraded after protease treatment. However, if this is not seen, it is likely that one is dealing with aggregated proteins. In order to get an indication if the imported proteins are membrane-integrated, unspecifically bound precursor proteins are removed by protease treatment (if possible) and mitochondria are subsequently incubated in 0.1 *M* Na_2CO_3. Under these conditions, most integral membrane proteins remain in the membrane pellet while soluble proteins are released into the supernatant. In cases where a wild-type yeast mitochondrial protein is imported, it is recommended to check by immuno-decoration that the endogenous protein shows the identical behavior with regards to protease accessibility and membrane extractability.

1. Perform a 700-µL import reaction and move it on ice.
2. Take 600 µL of the import reaction, spin (5 min, 12,000*g*, 2°C), and resuspend the mitochondrial pellet in 60 µL SEM.
3. Add 20 µL to 180 µL SEM, EM, or SEM + 0.5% (w/v) Triton X-100, respectively, mix by pipetting the whole volume 10 times carefully up and down with a yellow tip, and incubate 15 min on ice.
4. Split these samples into 2 × 95 µL, add 2.5 µL 1 mg/mL Proteinase K to one-half, and incubate 15 min on ice.
5. Add 1 µL PMSF to all samples, mix on a vortex at low speed, and incubate for 10 min on ice. Spin (5 min, 12,000*g*, 2°C), carefully take the supernatant off (*see* **Note 13**) and precipitate the proteins from the supernatant of the SEM + Triton X-100 samples (e.g., with StrataClean, Stratagene, or TCA; Trichloroacetic acid). Wash the mitochondrial pellets with SEM buffer containing 2 m*M* PMSF (do not resuspend the mitochondrial pellet) and spin (5 min, 12,000*g*, 2°C) again.
6. Take 95 µL of the import reaction from **step 1** and add 2.5 µL 1 mg/mL Proteinase K, mix, and incubate for 15 min on ice. Add 1 µL PMSF, mix, and incubate for 10 min on ice. Spin (5 min, 12,000*g*, 2°C) and carefully take the supernatant off (*see* **Note 13**). Wash the mitochondrial pellets with SEM buffer containing 2 m*M* PMSF (do not resuspend the mitochondrial pellet), spin (5 min, 12,000*g*, 2°C), resuspend the pellet thoroughly in 200 µL 0.1 *M* Na_2CO_3, and incubate 30 min on ice.
7. Spin (30 min, 200,000*g*, 2°C), take the supernatant off, and precipitate the proteins (e.g., with StrataClean, Stratagene, or TCA; *see* **Note 15**).
8. Load the pellets and the precipitated proteins of the supernatants from **steps 5** and **7** on an SDS-PAGE and analyze with a Phosphor Imager (*see* **Fig. 3**).

4. Notes

1. Many proteins are more efficiently translated if the RNA is capped with diguanosine triphosphate. Therefore, we add an excess of cap over GTP so that

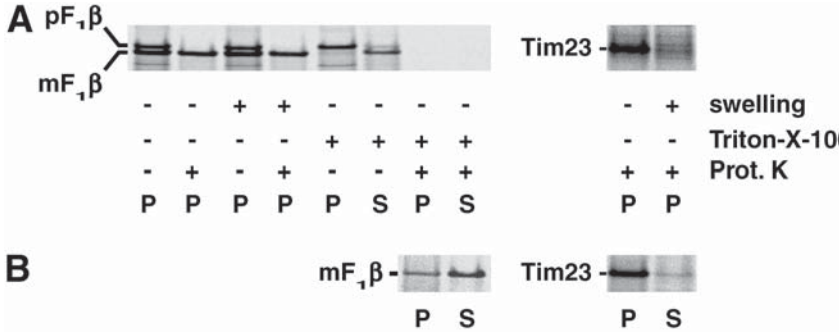

Fig. 3. Analysis of the in vitro import of $F_1\beta$ and Tim23 (pellet, P; supernatant, S). **(A)** Test of the localization of imported proteins. After the import reaction the mitochondria were either left untreated, swollen or lysed with Triton X-100. Half of each sample was subsequently treated with Proteinase K (Proteinase K). The matrix protein $F_1\beta$ and the integral inner membrane protein Tim23, with its intermembrane space domain, are both protected against proteolysis by the outer membrane. Only unspecifically bound precursor of $F_1\beta$ is degraded. After rupturing the outer membrane by swelling, the mature $F_1\beta$ remains protected by the inner membrane. However, the intermembrane space domain of Tim23 is degraded. After lysis of both mitochondrial membranes by detergent, mature $F_1\beta$ is accessible to protease and degraded. **(B)** Test for membrane integration of the imported proteins. After import, the unspecifically bound precursors were degraded by protease treatment. The mitochondria were resuspended in 0.1 *M* Na_2CO_3 and incubated on ice. Most integral membrane proteins are resistant to carbonate extraction and remain in the membrane pellet, while other proteins are found in the supernatant. The peripheral membrane protein $F_1\beta$ is predominantly detected in the supernatant, whereas the integral membrane protein Tim23 remains in the membrane pellet.

most of the resulting RNA is capped. However, a considerable number of proteins are equally well-translated without a cap. In this case, one can omit the (expensive) cap from the transcription reaction (*see* **Subheading 3.1., step 2**), but must also stimulate the transcription reaction by using the same amount of GTP as that of other nucleotides. In this case, one has to set up a transcription premix (*see* **Subheading 2.2.**) using 20 µL 0.1 *M* GTP.

2. It is possible to prepare reticulocyte lysate yourself ***(11)***, but the quality of the commercial lysate is more consistent. We prefer the Amersham rabbit reticulocyte lysate because of the option to adjust the salt concentrations. We have also had good experiences with the Promega Quick Transcription/Translation System. Yeast lysate is not commercially available and wheat germ or *Escherichia coli* lysate do not work in many cases for in vitro import reactions into yeast mitochondria.

3. Even though salt is typically used at physiological concentration, there are examples of precursor proteins for which the in vitro import can be stimulated by employing higher salt concentrations (up to 250 m*M* KCl).
4. Use of the the SP6 promotor usually yields good translation products. However, if one wants to express the protein in bacterial translation systems, it is worth it to try a T7 expression vector.
5. Alternatively, perform a polymerase chain reaction (PCR) reaction using a 5' primer containing an SP6 or T7 promotor (for SP6: 5' G GAT TTA GGT GAC ACT ATA GAA TAC **ATG** $N_{4\text{-}15/18}$, for T7: 5' T CTA ATA CGA CTC ACT ATA GGG AGA **ATG** $N_{4–15/18}$, where N stands for nucleotides downstream of the start codon) and a 3' primer that is reverse complement to the coding strand of the template DNA, either downstream of or spanning the stop codon. For the generation of N-terminal deletions, design the 5' primer such that N stands for the first 5 or 6 codons of the nucleotide sequence of the desired deletion construct. To generate C-terminal deletions, design the 3' primer reverse complement to the 5 or 6 last codons of the desired deletion construct plus stop codon TAA (e.g., 5' **TTA** $M_{15–18}$, where M stands for the reverse complement of the last nucleotides of the desired construct). Purify the PCR product (e.g., by phenol chloroform extraction), or in the case where nonspecific PCR products are generated, separate these by agarose gel electrophoresis and excise the band (e.g., Gel Extraction Kit, Qiagen). Resuspend the PCR product in RNAse-free H_2O and add 0.5 µg of PCR product instead of the plasmid DNA to the transcription reaction ***(12)***.
6. In some cases it helps to use a linearized vector, but usually this is not necessary.
7. To increase the yield, add an additional 2 µL of RNA polymerase (50 U/*m*L) and incubate for another hour.
8. It is very important to avoid drying the RNA pellet too long, because the solubilization of RNA can be reduced if it is too dry and this will eventually lower the efficiency of the translation reaction.
9. If the radiolabeling of the precursor protein is weak, the amount of RNA for the translation reaction should be increased as a first attempt to optimize transcription/translation. However, too much RNA can also reduce the efficiency of translation.
10. By varying the salt concentrations (Mg^{2+}, K^+), the efficiency of the translation reaction can be increased in some cases. However, it can considerably change the amount of internal starts of translation, although these secondary initiations are often unproblematic because they lack parts of the targeting signal and thus are not imported. The translation reaction with the commercial lysate can be left for longer times at 30°C, because proteolysis of the labeled protein seems to be no problem; however, this does not result in a considerably higher yield.
11. In case the translation reactions were limited by the amount of ^{35}S-methionine, add unlabeled methionine at the end of the incubation and incubate the reaction mix for an additional 5 min at 30°C to ensure that the translation reaction finishes with all radioactively labeled products.
12. It is very important for the integrity of the outer membrane to thaw the mitochondria on ice, which, depending on the size of the aliquot, can take up to 30 min.

13. After PMSF treatment the pellet is especially loose, so use extra care.
14. MOPS/met is added to dilute the radiolabeled unincorporated ^{35}S-methionine that is present in the lysate with cold methionine. If import reactions with chemical amounts of cold precursor proteins are performed, omit MOPS/met.
15. TCA precipitation of proteins in highly alkaline buffer are problematic and usually require higher TCA concentrations.

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