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PRINS and *In Situ* PCR Protocols

SECOND EDITION

Edited by

Franck Pellestor

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PRINS and *In Situ* PCR Protocols

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Second Edition

Edited by

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Dedication

This book is dedicated to the memory of our friend, Jean Paul Charlieu, a talented researcher and a pioneer of the PRINS technique, who disappeared in December 2001.

Preface

The *in situ* hybridization and PCR technologies are now well-established molecular techniques for studying chromosomal aneuploidy and rearrangements, gene localization and expression, and genomic organization. Over the last decade, we have seen increasing applications in these fields.

By combining the high sensitivity of the PCR reaction and the cytological localization of target sequences, both PRINS and *in situ* PCR techniques have provided highly powerful complements to FISH for *in situ* cellular and molecular investigations. Both these approaches have several advantages in terms of sensitivity and specificity, owing to the use of primers and to the fast kinetics of annealing and elongation reactions *in situ*.

In the first edition of *PRINS and* In Situ *PCR Protocols* edited by John R. Gosden, experts in the field presented in detail a variety of applications of PRINS and *in situ* PCR techniques, in a wide range of clinical conditions. Since the publication of this successful reference book, there have been significant improvements in *in situ* detection techniques.

This completely revised and updated second edition presents a comprehensive selection of new procedures developed in the field of PRINS and *in situ* PCR technologies. The book has two sections. Part I, Basic Methodology, contains chapters that provide useful protocols for many variations of PRINS and *in situ* PCR, including a new fast multicolor PRINS method, and protocols for PRINS detection of unique sequences *in situ*.

All these methods have been shown to be reliable procedures in the authors' laboratories, and the chapters display helpful notes for optimizing the procedure and avoiding pitfalls. Part II, Research and Clinical Applications, addresses particular applications of both PRINS and *in situ* PCR in research and diagnosis. The use of PRINS is described in various applications in humans (detection of gene deletions in cancer, detection of fetal cells in maternal blood, assessment of aneuploidy in brain tissues, in embryos), as well as in plant cells. A selection of *in situ* PCR techniques is also presented step by step (detection of cytomegalovirus, use of RT *in situ* PCR on plants or in cancer investigation, combination with microdissection), based on the experience of well-versed researchers and clinical investigators. All these chapters are focused on the practical aspects, introducing the reader to the background and operating procedures, and with a Notes section highlighting potential problems and providing hints and tips for success. Finally, the book closes with two

overviews on PRINS and *in situ* PCR technologies that discuss the latest developments in the field, as well as challenges for the foreseeable future.

Intended for molecular biologists, cytogeneticists, and cytologists, my wish would be that this book could be helpful to experts as a source of information, as well as to non-experts as a means of orientation in the rapidly developing field of *in situ* labeling technology.

Franck Pellestor

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Contents

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List of Color Pages

Color pages to follow p. 48

Color Page 1. *Fig. 2, Chapter 1:* Triple-color PRINS by omitting the blocking step for simultaneous detection of three different chromosomes in a normal sample using the bio-dig-bio labeling order and avidin-fluorescein/anti-dig-rhodamine system. (*See* full caption on p. 6 and discussion on p. 4.)

Color Page 2. *Fig. 3, Chapter 1:* Human telomere signals detected by double-strand PRINS. (*See* full caption on p. 7 and discussion on p. 4.)
Fig. 1, Chapter 2: Combined PRINS and PNA labeling on a chromosome spread. (*See* full caption on p. 20 and discussion on p. 19.)

Color Page 3. *Fig. 1, Chapter 5:* Rolling-circle PRINS from padlock probes. (*See* discussion on p. 42.)

Color Page 4. *Fig. 2, Chapter 5:* Detection of a gene in the long arm of human chromosome 6. (*See* full caption and discussion on p. 45.)

Color Page 5. *Fig. 1, Chapter 6:* Principles of the multicolor PRINS reaction (modified from Yan ***[10]***). (*See* full caption on p. 51 and discussion on p. 50.)

Color Page 6. *Fig. 2, Chapter 6:* Examples of *in situ* PRINS labeling of human sperm nuclei. (*See* full caption on p. 57 and discussion on p. 56.)

Color Page 7. *Fig. 1, Chapter 11:* **(A)** Example of *DMD* gene and X centromer labeling in chromosome and nucleus using PRINS and FISH protocols. (*See* full caption on p. 117 and discussion on pp. 117 and 118.)
Fig. 1, Chapter 14: Dual-color PRINS labeling on chromosome-X (red signal) and Y (green signal) of a fetal nucleus from maternal blood from a woman carrying a male fetus. (*See* full caption and discussion on p. 147.)

I

Basic Methodology

1

New Rapid Multicolor PRINS Protocol

Ju Yan, Macoura Gadji, Kada Krabchi, and Régen Drouin

Summary

In the multiple-color primed *in situ* labeling (multi-PRINS) technique, using ddNTPs between two PRINS reactions can block the free 3'-end generated in the previous PRINS reaction, thus avoiding the next PRINS reaction, using it as a primer to perform spurious elongation at nondesired sites. However, by omitting the blocking step and taking advantage of the color mixing, we developed a simple and rapid multi-PRINS technique to simultaneously detect three chromosomes in the same cell. With this protocol, one can create a third color using the two most common forms of labeled dUTP (biotin- and digoxigenin-labeled dUTP) and two fluorochromes (fluorescein and rhodamine). The signals at the centromeres of three different chromosomes displayed perfect yellow, red, and green colors, respectively. The entire procedure could be completed in less than 90 min because the blocking step was omitted. This protocol is practical and efficient for multi-PRINS so that even more than three chromosome targets could be detected in the same cell.

Key Words: Primed *in situ* labeling; multi-PRINS; cytogenetic techniques; molecular cytogenetics; human chromosomes.

1. Introduction

Using sequential labeling of different chromosome targets, the multiple color primed *in situ* labeling (multi-PRINS) technique can simultaneously and specifically display different chromosomes with different colors in the same metaphase or interphase nucleus ***(1–5)***, which can be particularly useful when a rapid screening for chromosomal abnormality is desired and the number of analyzable metaphases is limited. The first procedure developed to specifically identify different chromosome targets required a blocking treatment between two standard PRINS reactions. The incorporation of dideoxynucleotide triphosphates (ddNTPs) acts to block the free 3'-end of the DNA strands generated during the previous PRINS reaction, thus avoiding the subsequent PRINS

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reaction from using it as a primer to perform spurious elongation at nondesired sites ***(3,4,6)***. In practice, however, the blocking procedure does not always seem effective. In 1996, Coullin et al. ***(7)*** reported a similar technique to PRINS called HISOMA (hybridization of labeled oligonucleotides). In their technique, they used specifically labeled primers to study the α-satellite DNA of chromosome 1. This technique has been performed successfully on cytogenetic preparations from human heteroploid and human x hamster hybrid cell lines in addition to frozen tissue sections. The use of different fluorochromes and the possible combination with an unlabeled elongation in 3' of the oligonucleotides, which stabilize its hybridization, led to a simple multicolor method ***(7)***.

In the double-PRINS technique, to specifically detect two different chromosomes without the blocking step, we found that the correct identification easily can be performed because the signals obtained from the last PRINS reaction always show a single original color. From this finding, we developed a multi-PRINS technique ***(8)*** by omitting the blocking step and taking advantage of mixing two fluorochromes (fluorescein and rhodamine) to create a third color for the detection of three different chromosome targets (**Figs. 1** and **2**). By selecting the labeling order, either in bio-digoxigenin (dig)-bio or in dig-bio-dig order, in the sequential PRINS reaction, then detecting with a mixture of avidin-fluorescein/anti-dig-rhodamine or a mixture of anti-dig-fluorescein/avidin-rhodamine, the signals at the centromeres of three different chromosomes displayed perfect yellow, red, and green colors, respectively. Because the blocking step is omitted, the entire procedure can be completed in less than 90 min. We showed that this is a practical and efficient way to conduct multi-PRINS so that even more than three chromosome targets could be detected in the same cell.

This technique has been used successfully to determine the copy numbers of different chromosome targets from interphase nuclei in peripheral blood and bone marrow ***(9,10)*** and in amniotic fluid cells (Gadji et al., unpublished data). We also have recently developed a double-strand PRINS technique ***(11)***. A high labeling efficiency of human telomeres was obtained by using two primers, $(TTAGGG)_7$ and $(CCCTAA)_7$, to label both forward and reverse telomeric DNA strands (**Fig. 3**). On the basis of the principle of this multi-PRINS technique, four sequential multi-PRINS reactions were performed successfully on spermatozoa, lymphocytes ***(12)***, oocytes, and polar bodies ***(13)***. The following fluorochromes fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), and Cascade Blue incorporated in sequential PRINS reactions allowed the rapid and distinct labeling of four chromosomes displaying distinct color spots (green, red, yellow, and blue).

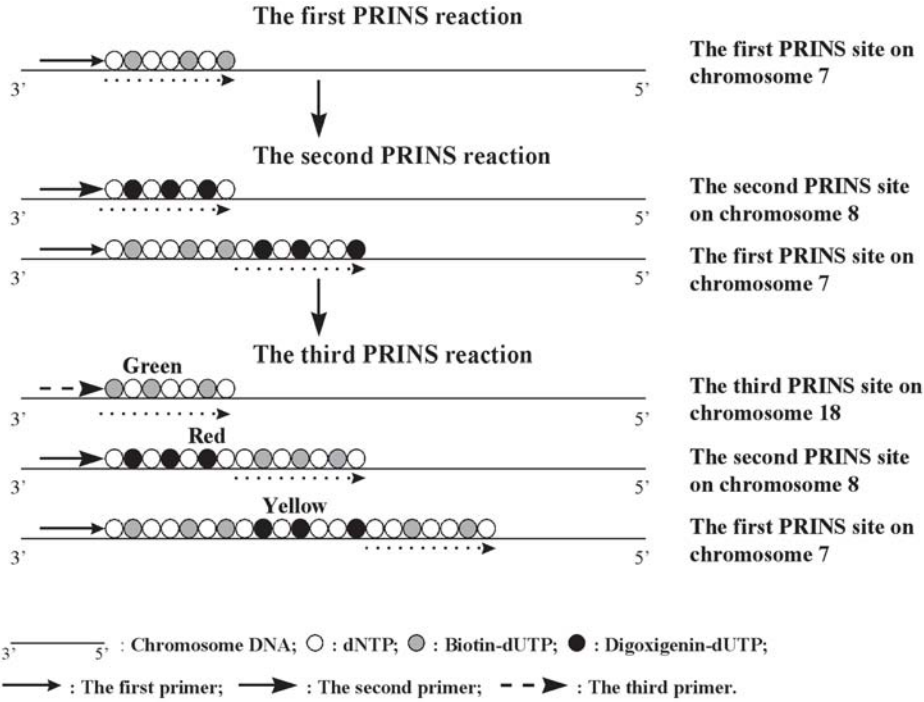

Fig. 1. The schematic triple-color PRINS technique. After three sequential PRINS reactions, the target sites of chromosomes 7, 8, and 18 were identified with yellow, red, and green colors, respectively. Horizontal dashed arrows under the chromosomal DNA indicate each strand extension site from the beginning to the end of each PRINS reaction. The procedure illustrated corresponds to the bio-dig-bio labeling order using the detection mix avidin–fluorescein/anti-dig–rhodamine. A similar schema can be used to illustrate the reverse labeling order, dig-bio-dig, using the detection mix anti-dig–fluorescein/avidin–rhodamine ***(8)***.

2. Materials

2.1. Preparation of Samples

1. Cell suspension of metaphases and interphase nuclei obtained from a routine cytogenetic procedure.
2. Precleaned microscope glass slides and cover slips.
3. 70%, 80%, and 100% ethanol.
4. Thermotron environmental control unit (CDS-5, Thermotron, Amsterdam, Holland).
5. 20X standard saline citrate (SSC): 3.0 *M* NaCl, 0.30 *M* trisodium citrate, pH 7.2–7.4.

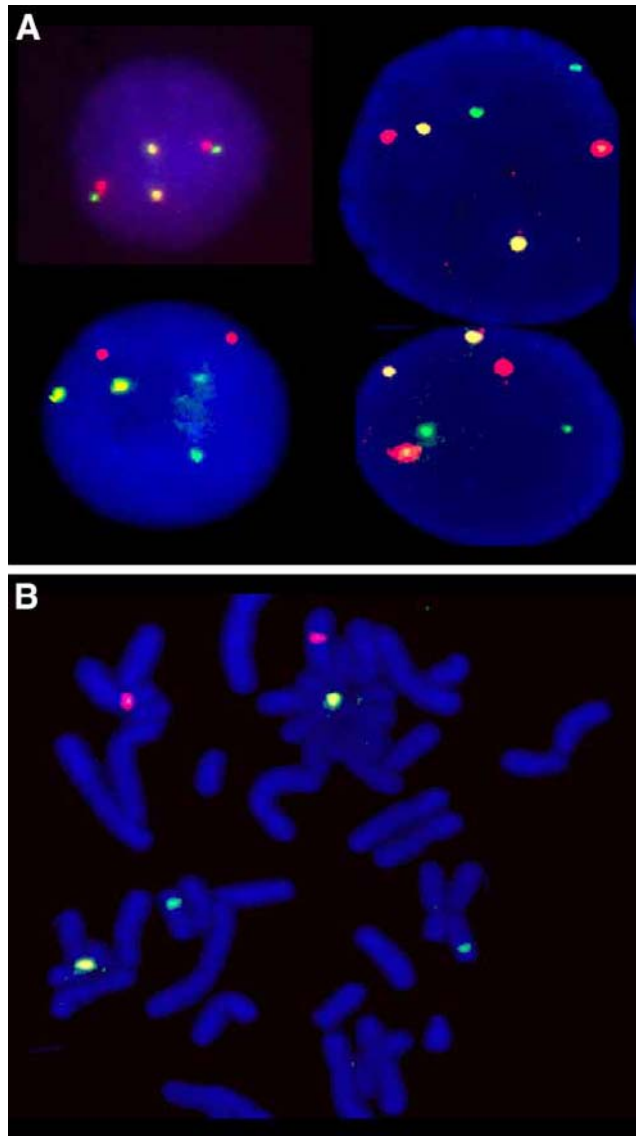

Fig. 2. Triple-color PRINS by omitting the blocking step for simultaneous detection of three different chromosomes in a normal sample using the bio-dig-bio labeling order and avidin-fluorescein/anti-dig-rhodamine detection system. The yellow signals in nuclei (**A**) and a metaphase (**B**) correspond to the first PRINS target site at the centromere of chromosome 7. The red signals were generated by the second PRINS target and correspond to the site of the centromere of chromosome 8. The pure-green signals correspond to the third PRINS site located at the centromere of chromosome 18 ***(8)***. (Please *see* color insert following p. 48.)

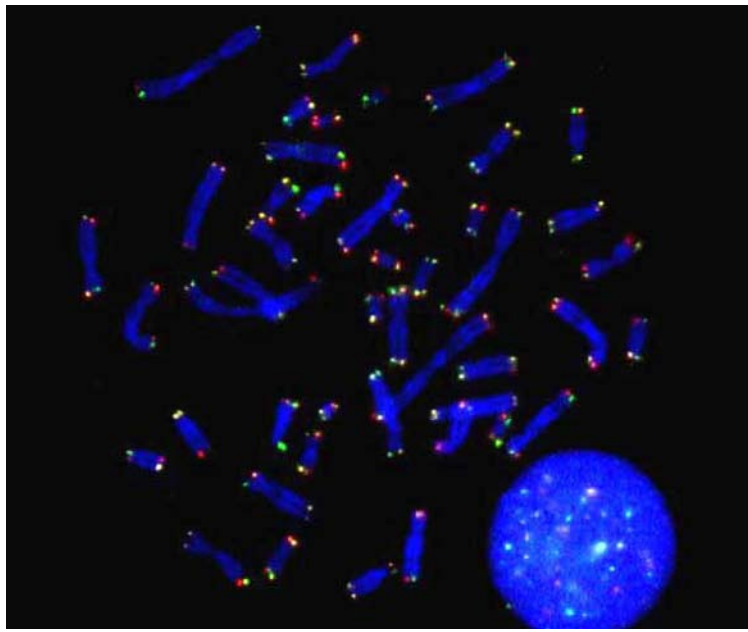

Fig. 3. Human telomere signals detected by double-strand PRINS. The number of telomere signals is very high when using the dual-color PRINS. This typical metaphase spread shows that three signal patterns can be identified. The green signal pattern was generated only from $(TTAGGG)_7$ primer extension and labeled by biotin–dUTP, the red signal pattern was generated only from $(CCCTAA)_7$ primer extension and labeled by digoxigenin–dUTP, and the combined green and red signal pattern (yellow) was generated from extension of both complementary primers ***(11)***. (Please *see* color insert following p. 48.)

6. 2X SSC (diluted from 20X SSC), pH 7.2–7.4. Store the SSC solutions at ambient temperature and discard after 6 mo or sooner if the solution appears cloudy or contaminated.
7. TE buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* ethylene diamine tetraacetic acid (EDTA) (*see* **Note 1**).
8. Denaturation solution: 70% formamide/2X SSC.
9. Water bath set at 70°C.
10. Incubator set at 37°C.
11. Microtubes (1.5 mL).

2.2. PRINS Reactions

1. Primer stock solution: this solution is prepared by dissolving each primer (*see* **Table 1** for the sequence) in TE buffer for a final concentration of 100 µ*M* (*see* **Note 1**).

Table 1
Primers That Have Been Used in Multi-PRINS Technique

Name	Location	Sequence	Annealing temperature	References
Alpha 7	7	GCTTGAAATCTCCACCTGAAATGCCACAGC	62.5°C	*1*
8c	8	CTATCAATAGAAATGTTCAGCACAGTT	62.5°C	*3*
18c	18	ATGTGTGTCCTCAACTAAAG	62.5°C	*14*
Xc	X	GTTCAGCTCTGTGAGTGAAA	65°C	*14*
D599	Y	TGGGCTGGAATGGAAAGGAATCGAAAC	56°C	*2*
D600	Y paired with D599	TCCATTCGATTCCATTTTTTTCGAGAA	56°C	*2*
Tel. 1	Telomere	$(TTAGGG)_7$	62.5°C	*15*
Tel. 2	Telomere	$(CCCTAA)_7$	62.5°C	*15*

2. dNTP working solution: Dilute 1 µL each of 100 m*M* stock solution of dCTP, dGTP, and dATP (Roche Molecular Biochemicals) with 39 µL of sterile distilled water to a concentration of 2.5 m*M*. Dilute 100 m*M* stock solution of dTTP (Roche Molecular Biochemicals) to a concentration of 0.25 m*M* by mixing 1 µL of dTTP with 399 µL of sterile distilled water.
3. 1 m*M* of biotin-16-dUTP or biotin-11-dUTP (Enzo), and dig-11-dUTP (Roche Molecular Biochemicals).
4. *Taq* DNA polymerase (Roche Molecular Biochemicals).
5. 10X polymerase chain reaction (PCR) buffer (Roche Molecular Biochemicals).
6. 10X phosphate-buffered saline (PBS): 1360 m*M* NaCl, 20 m*M* KCl, 106 m*M* Na_2HPO_4, 15 m*M* KH_2PO_4, pH 7.2–7.4.
7. 1X PBS, pH 7.2–7.4 (diluted from stock 10X PBS). Store PBS solutions at ambient temperature and discard after 6 mo or sooner if the solution appears cloudy or contaminated.
8. Washing buffer: 4X SSC (diluted from stock 20X SSC), 0.05% Triton X-100, or 0.2% Tween-20.
9. Blocking buffer: Washing buffer with the addition of 5% skimmed milk powder.
10. Thermal cycling machine equipped with a flat block (PTC-100, MJ Research; *see* **Note 2**).

2.3. Detection

1. 1% Avidin-fluorescein DCS (Vector Laboratories, Burlingame, CA) and 1% anti-dig-rhodamine (Roche Molecular Biochemicals; diluted with blocking buffer).
2. 1% Avidin-rhodamine (Roche Molecular Biochemicals) and 1% anti-dig-fluorescein (Roche Molecular Biochemicals; diluted with blocking buffer). Either of two detection mixes above can be used according to the selected labelling protocol for multiple-chromosome target detection (*see* **Note 3**).
4. Counterstaining solution: In 1 mL of 0.1 *M* Tris-HCl, pH 7.0, dissolve 125 ng of DAPI (Sigma), 1 mg of *p*-phenylenediamine, and then mix with 9 mL of glycerol.
5. Fluorescence microscope equipped with appropriate filters and connected to an image system is needed (e.g., ISIS 2 in MetaSystems, Belmont, MA).

3. Methods

3.1. Preparation of Samples

1. Drop the cell suspension onto precleaned slides. Thermotron conditions should be 25°C and 50% humidity (*see* **Notes 4** and **5**).
2. Incubate the slides in 2X SSC for 15 to 30 min at 37°C and then dehydrate the slides through a series of ethanol (70%, 80%, and 100%) at room temperature. For amniotic cells, a specific pretreatment is applied (*see* **Note 6**).
3. Prepare 50 mL of denaturation solution (70% formamide/2X SSC) by mixing thoroughly 35 mL of formamide (ultrapure grade, Fluka), 5 mL of 20X SSC, and 10 mL of distilled water in a glass Coplin jar at a pH value of 7.2–7.4. Before use, prewarm the solution until it reaches 70°C. Between uses, store denaturation solution at 2 to 8°C and discard it after 3 to 5 d.

4. Denature slides in 70% formamide/2X SSC at 70°C for 2 min and successively pass them through 70%, 80%, and 100% ethanol for 2 min each at –20°C. After air-drying, the slides are ready for the PRINS procedure.

3.2. PRINS Reactions

1. Mix 5 µL of primer stock solution with 95 µL of TE buffer or water for a 5 µ*M* primer working solution.
2. For each slide, mix in a 1.5-mL microtube:
   a. 5 µL of 10X PCR buffer.
   b. 4 µL of each dNTP working solution, a total of 16 µL.
   c. 2 µL of primer working solution.
   d. 1 µL of 1 m*M* biotin-dUTP or 1 µL of 1 m*M* dig-dUTP.
   e. 2.5 µL of glycerol (*see* **Note 7**).
   f. 23 µL of distilled water.
   g. 0.5 µL of *Taq* DNA polymerase (add immediately before starting the PRINS reaction).

3.2.1. Double PRINS

1. Initiate the PRINS reaction by adding the first reaction solution (50 µL) containing a specific chromosome primer and one of the labels onto a slide. Cover the slide with a cover slip and put the slide on the flat block of the thermocycler.
2. Perform annealing and extension steps according to the different primers used for the detection of different chromosome targets (*see* **Note 8**).
3. Wash the slide briefly in 1X PBS solution for 2 min after the first PRINS reaction.
4. Add the second reaction solution containing a primer specific for the second chromosome target and the other labeled dUTP on the same slide.
5. Perform the second PRINS reaction as **step 2**.
6. Wash the slide in washing buffer for 5 min with gentle agitation (*see* **Note 9**).

3.2.2. Triple PRINS

1. Perform double PRINS with two different labels for two different chromosome targets as mentioned previously.
2. Following **step 5** of **Subheading 3.2.1.**, wash the slide briefly in PBS solution for 2 min and add the third PRINS reaction solution containing a primer specific for the third chromosome target on the same slide. Use a labeled dUTP that is the same as the one used in the first PRINS reaction.
3. Perform the third PRINS reaction under an appropriate condition of annealing and strand elongation according to the primer used (*see* **Note 8**).
4. Wash the slide in washing buffer for 5 min with gentle agitation (*see* **Note 9**).
5. Drain the washing buffer from the slide and quickly go to the detection steps.

3.3. Detection

1. Mount the slide with 100 µL of blocking buffer and a cover slip. Leave the slide at room temperature for 5 min.

2. Remove the cover slip, drain blocking buffer from the slide, and apply 100 µL of a detection solution mix (*see* **Note 10**) to the slide.
3. Place a cover slip and incubate the slide in a moist chamber at 37°C for 30 min.
4. Remove the cover slip and wash the slide three times for 5 min each in washing buffer at room temperature with gentle agitation.
5. As soon as the slide has air-dried in the dark, apply 10 to 20 µL of DAPI counterstaining solution to the slide and cover the slide with a cover slip.
6. Examine the slide under the fluorescence microscope and image system.

4. Notes

1. The lyophilized oligonucleotide is stable at –20°C for 1 yr or longer. It is generally accepted that oligonucleotides dissolved in TE are stable for at least 6 mo at –20°C or 4°C. Oligonucleotides dissolved in water are stable for at least 6 mo at –20°C. Do not store oligonucleotides in water at 4°C. TE is recommended compared with deionized water because the pH value of the water is often slightly acidic and can cause oligonucleotide hydrolysis.
2. In our experience, the MJ Research Thermocycler (PTC-100) gives us the best results. The Hybrid from Vysis Inc can be used, but the temperature program variation should be validated to perform multi-PRINS adequately.
3. In the detection solution, a relatively weak fluorochrome, for example, avidin–fluorescein or anti-dig–fluorescein (green), should be used for the last PRINS target labeling to minimize the color mixing in the previous PRINS target. Whereas the first labeling uses biotin-dUTP and the second labeling uses dig-dUTP (bio-dig) for two different chromosome targets, respectively, an appropriate fluorochrome mix should be avidin–rhodamine/anti-dig–fluorescein. Conversely, if the labeling order is dig-bio, the fluorochrome mix should be anti-dig–rhodamine/avidin–fluorescein. The principle of selecting a relatively weak fluorochrome for the last PRINS target detection is critical for double PRINS. For the triple PRINS reaction, the weak fluorochrome should be used to detect the first and last PRINS targets.
4. The efficiency of the PRINS relies greatly on the temperature conditions and the humidity when preparing slides. The optimal conditions for the dropping of cells onto slides can be reached by using a Thermotron (CDS-5) ***(16)*** or in a temperature/humidity-adjustable chamber. The best conditions for PRINS may vary from laboratory to laboratory and should be determined by testing beforehand. Ideally, the nuclei on the slide should show a gray color, and no reflective nuclei or bright rings around any nuclei should be observed.
5. The PRINS reaction works optimally with freshly prepared slides. The slides should be completely dry and are then ready for PRINS. However, slides stored at –20°C for up to 1 mo can be used for the PRINS reaction.
6. For uncultured amniotic cells, necessary pretreatment is performed by incubating the slides in formaldehyde–PBS/$MgCl_2$ solution (100 mL PBS/50 m*M* $MgCl_2$ and 2.7 mL 37% formaldehyde) for 1 min at room temperature, followed by rinsing in 1X PBD buffer (0.1 *M* NaH_2PO_4/0.1 *M* Na_2HPO_4 and 0.1% Nonidet) for 1 min before the dehydration step using an ethanol series.

7. 5% Glycerol can effectively prevent the drying of the PRINS reaction solution during the annealing and extension steps of PRINS on the heating block of the themocycler. Higher concentrations of glycerol may cause inactivation of DNA polymerase.
8. A single step of primer annealing and strand extension for most primers was performed at 62.5°C for 15 min. For X chromosome detection, the PRINS program is as follows: annealing at 65°C for 10 min and extension to 72°C for 10 min. For the Y chromosome, annealing is at 56°C for 10 min and extension is at 72°C for 10 min.
9. Washing at room temperature for 5 min usually is sufficient. In the case of high background interference, the slide can be washed once at 45°C for 5 min and twice at room temperature for 5 min.
10. When the labeling order is bio-dig-bio, a 100-µL mix of 1% avidin–fluorescein DCS and 1% anti-dig–rhodamine is used; however, when the labeling order is dig-bio-dig, a 100-µL mix of 1% anti-dig–fluorescein and 1% avidin–rhodamine is used. In triple PRINS, a new color, yellow, is created for the first PRINS target because it mixes the green labeling twice and the red labeling once (**Fig. 1**).

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2

PRINS Combined With Peptide Nucleic Acid Labeling

Franck Pellestor and Petra Paulasova

Summary

Both primed *in situ* labeling (PRINS) and peptide nucleic acid (PNA) technologies have emerged as research techniques, but they have quickly evolved to applications in biological diagnosis assays. The two procedures present several features (specificity, discriminating ability, rapidity) that make them very attractive for cytogenetic purposes. The combined use of PRINS and PNA for *in situ* chromosomal detection on a same cell preparation is described in this chapter.

Key Words: PRINS; PNA; *in situ* labeling; chromosomes; aneuploidy.

1. Introduction

Both the primed *in situ* (PRINS) labeling and the peptide nucleic acid (PNA) techniques constitute alternatives to the fluorescence *in situ* hybridization (FISH) for chromosomal screening and aneuploidy detection.

The PRINS labeling is based on the use of short specific primers for repeated DNA sequences. An advantage of primers is their ability to differentiate between closely related sequences ***(1,2)***. This feature has been used for generating chromosome-specific primers from the centromeric α-satellite DNA motifs ***(3,4)***. Since its introduction, the PRINS procedure has been considerably improved and simplified, with the development of sequential multicolor protocols and the direct use of fluorochromes. Numerous applications of PRINS have been described in humans, mammals, fish, insects, and plants, demonstrating that the PRINS procedure can be easily adapted to various types of cells ***(5–7)***.

The PNAs are a new class of probes recently introduced in molecular cytogenetics. These molecules are synthetic nucleic acids analogs in which the phosphodiester backbone is replaced by a noncharged peptide-like backbone ***(8)***. This unique structure gives PNAs the capacity to hybridize to complemen-

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tary RNA and DNA sequences with high affinity and specificity and a great resistance to nucleases and proteinases. The remarkable physicochemical properties of PNAs have led to the development of a large variety of research and diagnostic assays, including antigene and antisense therapy, genome mapping, and mutation detection ***(9)***. During the last few years, the use of PNAs has proven its powerful usefulness in cytogenetics. Recent studies have reported the successful use of chromosome-specific PNA probes on human lymphocytes, amniocytes, and spermatozoa, as well as on isolated oocytes and blastomeres, indicating that PNAs could become a valuable tool for *in situ* chromosomal investigations ***(10–12)***.

PRINS and PNA present several features that make them very attractive for cytogenetic purposes. These two techniques can advantageously be combined on a same cell preparation. This combined use opens up interesting possibilities for multiplex assays. This chapter describes this innovative procedure for *in situ* detection of several chromosomes.

2. Materials

2.1. Slide Preparation

1. Metaphase preparations are prepared from mitogen-stimulated human blood lymphocytes using standard cytogenetic techniques (i.e., fixing them in methanol:glacial acetic acid 3:1 and spreading them on cleaned microscope slides). Slides must be used within a week of preparation and stored at room temperature until use (*see* **Note 1**).
2. Ethanol series: 70%, 90%, 100% (Prolabo, Paris, France).
3. 20X standard saline citrate (SSC): 3 *M* NaCl, 0.3 *M* trisodium citrate, pH 7.5 (can be stored for several months at room temperature).
4. Desionized formamide (Intergen Company, New York, NY), stored at 4°C.
5. Water bath at 73°C.
6. Light microscope Leica DMLB with ×10 and ×40 magnification (Leica France, Rueil-Malmaison, France).

2.2. PRINS Reaction

1. 2'-Deoxyadenosine 5'-triphosphate (dATP): 100 m*M* solution (Roche Diagnostics, Meylan, France) diluted 1:10 with sterile distilled H_2O.
2. 2'-Deoxycytosine 5'-triphosphate (dCTP): 100 m*M* solution (Roche Diagnostics) diluted 1:10 with sterile distilled H_2O.
3. 2'-Deoxyguanosine 5'-triphosphate (dGTP): 100 m*M* solution (Roche Diagnostics) diluted 1:10 with sterile distilled H_2O.
4. 2'-deoxythymidine 5'-triphosphate (dTTP): 100 m*M* solution (Roche Diagnostics) diluted 1:100 with sterile distilled H_2O.
5. Fluorescein-12-2'-deoxyuridine 5'-triphosphate (FITC-12-dUTP),1 m*M* (Roche Diagnostics).

6. Bovine serum albumin (BSA; Sigma, St. Louis, MO).
7. *Taq* DNA polymerase (Roche Diagnostics) or Ampli*Taq* (Perkin Elmer, Foster City, CA).
8. 10X *Taq* buffer: 500 m*M* KCl, 100 m*M* Tris-HCl, pH 8.3, 15 m*M* $MgCl_2$.
9. Oligonucleotide primer specific for chromosome 9, at 50 pmol/µL (*see* **Table 1** in Chapter 6).
10. Sterile distilled water.
11. Washing buffer: 2X SSC, diluted from 20X SSC in distilled water.
12. 1.5 mL of sterile microcentrifuge tubes (Eppendorf AG, Hamburg, Germany).
13. Cover slips (22 × 32 mm; CML, Nemours, France).
14. Coplin jar (50 mL).
15. Programmable thermal cycler equipped with a flat pate block (Hybaid Ltd., Teddington, UK; *see* **Note 2**).

2.3. PNA Reaction

1. The PNA probes are supplied ready to use in hybridization buffer (Applied Biosystems, Foster City, CA). Each PNA probe consists of a mixture of several short synthetic sequences (15–22 base units) specific for the centromeric repeated DNA sequence of the targeted chromosome. PNA probes specific for chromosomes 1 and 16 are used in the present protocol (*see* **Note 3**). The chromosome 1-specific probe is labeled in blue with diethlaminocoumarine. The chromosome 18-specific probe is labeled in red with rhodamine.
2. Phosphate-buffered saline (PBS; Gibco BRL, Eragny, France).
3. Tween-20 (Roche Diagnostics).
4. Washing buffers: 1X PBS, 0.1% Tween-20 and 2X SSC, 0.1% Tween-20.
5. 1.5 mL of sterile microcentrifuge tubes (Eppendorf AG).
6. Rubber cement (Artos, Strasbourg, France).
7. Cover slips (22 × 32 mm; CML).
8. Water bath set at 73°C.
9. Humidified hybridization chamber.
10. Coplin jar (50 mL).
11. Incubator set at 37°C.

2.4. Detection and Microscopy

1. 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI).
2. Propidium iodide (Sigma).
3. Antifade solution Vectashield (Vector Labs, Burlingame, CA).
4. Cover slips (20 × 40 mm; CML).
5. Rubber cement (Artos).
6. Epifluorescence Microscope Leica DMRB (Leica, France) equipped with ×40 and ×100 Plan FluoTar objectives and with a DAPI single band-pass filter (Leitz filter A, cat. no. 513804), an FITC single band-pass filter (filter I3, cat. no. 513808), a tetramethylrhodamine isothiocyanate (TRITC) single band-pass filter (filter N2.1, cat. no. 513812), an FITC/TRITC double band-pass filter (filter G/R,

cat. no. 513803), and a triple filter (filter B/G/R, cat. no. 513836) for simultaneous observation of DAPI/Cascade-Blue, FITC, and TRITC signals.
7. For image capturing, we use the software Metasystem Isis, Version 5.0 (Metasystem, Altlusshein, Germany).

3. Methods

3.1. Slide Preparation

1. Check the slides under the light microscope to ensure that both the cell concentration and the spreading are optimum.
2. Dehydrate the slides by passage through an ethanol series (70%, 90%, 100%) at room temperature, 3 min for each step, and air dry.
3. Denature chromosomal DNA by immersing the slides in 70% formamide, 2X SSC, pH 7.0, at 73°C for 4 min (*see* **Note 4**).
4. Pass slides through an ice-cold ethanol series (70%, 90%, 100%), 3 min for each step, and air dry.

3.2. PRINS Reaction

1. Prepare a reaction mixture in a final volume of 50 µL containing: 0.2 m*M* of dATP, dCTP, and dGTP; 0.02 m*M* of dTTP; 0.02 m*M* of FITC-12-dUTP; 50m*M* KCl, 10 m*M* Tris-HCl, pH 8.3; 1.5 m*M* $MgCl_2$; 0.01% BSA; 200 pmol of oligonucleotide primer; and 2.5 U of *Taq* DNA polymerase. In practice, mix in a sterile microcentrifuge tube: 1 µL of each 1:10 diluted dATP, dCTP and dGTP; 1 µL of the 1:100 diluted dTTP; 1 µL of FITC-12- dUTP; 1 µL of BSA; 5 µL of 10X *Taq* buffer, 0.5 µL of the *Taq* DNA polymerase, 4 µL of the primer specific for chromosome 9, and distilled water to 50 µL.
2. Place the reaction mixture under a 22 × 32 cover slip on the denatured slide and transfer to the heating block of the thermal cycler.
3. Set up the PRINS program for the appropriate temperatures and start the reaction. Two distinct programs can be used:
   a. The standard PRINS program consisting of two steps: 5 min at the annealing temperature (56°C for chromosome 9 primer according to **Table 1** in Chapter 6) and 5 min at 72°C.
   b. The fast PRINS program (according to **Subheading 3.2.1.**, **step 3** in Chapter 6) consisting of a unique 5 min step at the specific annealing temperature of the chromosome 9 primer (56°C).

While the reaction is running, prepare the PNA reaction mixture as described in **Subheading 3.3.**

4. On completion of the program, carefully remove the cover slip from the slide using a scalpel blade (avoid moving the cover slip across the chromosome spread).
5. Transfer the slide in a Coplin jar containing 2X SSC and wash the slide twice for 3 min at room temperature.

3.3. PNA Reaction

1. Prepare the PNA reaction mixture: aliquots of 5 µL of each PNA probe (chromosome 1- and chromosome 18-specific probes) are mixed into a microcentrifuge tube.
2. Denature the PNA probe mixture at 73°C for 6 min.
3. After draining the excess 2X SSC off the slide, and before the slide is completely dry, apply the PNA reaction mixture on the slide, and cover with a 22 × 32 cover slip.

No additional denaturation of the slide is required after the PRINS reaction because the chromosomal DNA remains denatured through the PRINS incubation.

4. Seal the slide with rubber cement (*see* **Note 5**).
5. Put the slide in a humidified hybridization chamber 60 min at 37°C.
6. At the end of the hybridization, carefully remove the cover slip from the slide using a scalpel blade.
7. Transfer the slide in a Coplin jar containing 1X PBS, 0.1% Tween-20, and wash the slide for 2 min at room temperature with gentle agitation.
8. Transfer the slide to 58°C prewarmed 1X PBS, 0.1% Tween-20 for 10 min with gentle agitation
9. Rinse the slide in 2X SSC, 0.1% Tween-20 for 1 min.

3.4. Detection and Microscopy

1. Drain the excess washing solution off the slide.
2. Mount the slide in Vectashield antifade solution containing a mix of propidium iodide (0.3 µL/mL) and DAPI (0.3 µL/mL). Use 15 to 20 µL of mountant/slide.
3. Cover with a 22 × 40 cover slip and seal the cover slip with rubber cement.
4. Examine the slide under the epifluorescence microscope equipped with suitable filters. Preferentially, use first the triple band-pass filter and confirm the coloring of the fluorescent spot with single band-pass filters. **Figure 1** shows typical results obtained on a human metaphase.

4. Notes

1. The age of slides is an important parameter. Slides should be used within a week of preparation. Best results in standard PRINS reaction are obtained with 2-d-old spreads because they give the best signals and chromosomal morphology. Using slides more than 1 to 2 wk old can be successful, but may lead to reduced sensitivity.
2. Several companies commercialize specialized thermal cyclers with flat block, for example, Techne Corporation (Cambridge, UK), MJ Research. (Watertown, MA), Hybaid, and Perkin Elmer. Because of differences in heat block design, technical conditions need to be optimized for the respective instrument used. Attaining accurate temperature at the top surface of the slide is crucial for PRINS

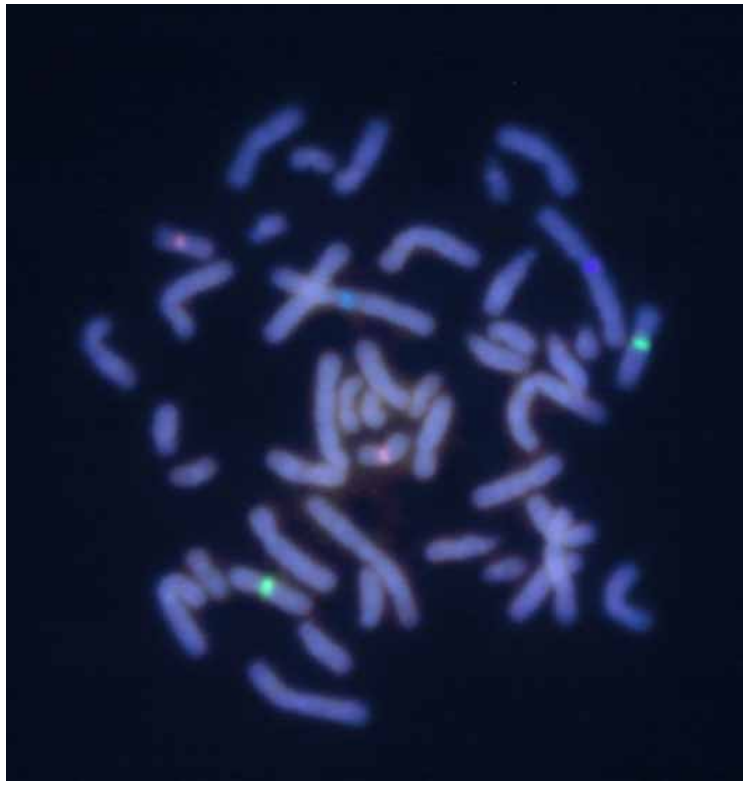

Fig. 1. Combined PRINS and PNA labeling on a chromosome spread. Chromosome 9 is labeled by PRINS in green. Chromosomes 1 and 18 are labeled by PNA probes, in blue and red, respectively. (Please *see* color insert following p. 48.)

reaction. Some programmable thermal cyclers, for example, the Hybaid Omnislide, possess incorporated control software to compensate for the temperature difference between the block and the surface of the slide.

3. PNA probes can be prepared after standard solid-phase synthesis protocols for peptides, but the production requires laboratories with the experience or the resources to support manual or automated peptide synthesis and consequently it is not easily accessible for cytogenetics laboratories. The commercial availability of PNA probes for cytogenetic purposes is still limited to consensus telomeric and a few human-specific satellite DNA probes. Until 2001, Boston Probes Inc. (Bedford, MA) was the leader in the development of PNA technology. In November 2001, the company was acquired by Applied Biosystems, which has pursued the development and the commercialization of PNA probes. A custom PNA probe service, PNA design guidelines, and a PNA probe order service are available on the Applied Biosystems web site (www.appliedbiosystems.com). DAKO A/S (Glostrup, Denmak), which was the majority owner of Boston Probe Inc., has always made available a commercialized consensus telomeric PNA

probe kit (www.dakocytomation.com). The PNA probes are compatible with a wide range of reporter molecules and fluorochromes, including fluoresceine and rhodamine, as well as cyanine and Alexa dyes available for a large variety of colors. The price of human chromosome PNA probe remains more expensive than the corresponding fluorescence *in situ* hybridization probes. However, one can hope that the success of the first generation of PNA probes will stimulate the future production of an extended variety of PNA probes and a decrease in their cost.

4. As an alternative, thermic denaturation can be used. In this case, apply the PRINS reaction mixture directly on the air-dried slide (after **step 2** in **Subheading 3.1.**), under a cover slip. Seal with rubber cement, air dry the rubber cement, and place the slide on the heating block of the thermal cycler. Set up the following program: 3 min at 94°C to ensure the denaturation of chromosomal DNA, followed by the standard PRINS program (**Subheading 3.2.**, **step 3a**), or the fast PRINS program (**Subheading 3.2.**, **step 3b**).
5. A seal is not required for PRINS reactions using chemical denaturation. Both the volume of the mixture and the short incubation time prevent the slide from drying out during the reaction. For PRINS with thermic denaturation (**Note 4**) or PNA hybridization, rubber cement provides an adequate seal that is easily and completely removed at the end of the reaction. Nail polish also provides a very secure seal but is more difficult to remove at the end of the procedure. It also is possible to use adhesive plastic frames and cover slips (e.g., Hybaid Sure Seal), which allow a "window" on the surface of the slide, to which one can spread the reaction mixture and ensure a good seal during the reaction. In all cases, care should be taken not to trap any air bubbles. Bubbles (including small ones) will expand during the reaction and strongly affect the quality of the labeling by creating on the slide large area without signals.

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3

PRINS Combined With Indirect Immunofluorescence

Philippe Coullin

Summary

Primed *in situ* labeling (PRINS) has proven to be an attractive alternative to fluorescence *in situ* hybridization for *in situ* DNA labeling and for being combined on the same slide with others methods. For the aim of a study on the asymmetrical segregation of the chromosomes of the megakaryocytes during polyploidization, we developed a simultaneous PRINS and immunofluorescent labeling. We report here the method, which enabled us to visualize the centromere of selected chromosomes and the achromatic spindle on the same picture, a method that may be of a more general application.

Key Words: PRINS; indirect immunofluorescence; centromere; spindle.

1. Introduction

The primed *in situ* labeling (PRINS) methodology, based on rapid annealing of primers followed by *in situ* elongation for chromosome labeling as proposed by Koch et al. ***(1)*** and Gosden et al. ***(2)***, could be combined with other methods such as fluorescent *in situ* hybridization (FISH ***[3]***). The simultaneous visualization of a DNA and a protein target is another goal. The combination of FISH and immunofluorescence offers multiple possibilities for research. However, the simultaneous generation of signals involving DNA and protein staining is difficult. FISH and protein immunostaining already have been described ***(4)***, and FISH and indirect immunoflorescence have been combined successfully ***(5,6)***. However, despite the widespread use of these techniques, they still require labor-extensive protocol adjustments to achieve correct and satisfactory simultaneous signal detection. Among the problems encountered, there are (1) the persistence of immunological signal after FISH treatments, (2) the permeability of DNA probe after protein fixation, and (3) the antigen durability after DNA denaturation.

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As the aim of a study on the chromosome distribution and segregation during the polyploidisation process of megakaryocytes ***(7)*** and after some wastage using FISH, we developed a method combining PRINS for labeling of selected centromeres with fluorescent immunostaining of tubulin for visualizing the spindle. We speculated that PRINS would prove more advantageous than FISH for several reasons: (1) the method is rapid, (2) the diffusion of small oligonucleotides within the material would be better than it is with classic FISH probes, (3) the prolonged contacts with formamide required during FISH incubation would be avoided, (4) PRINS is highly specific, and (5) PRINS had yet been successively combined with immunochemistry for the study of the organization of repetitive DNA sequences with respect to the kinetochore and for the detection of SV40-like viral DNA and antigens in malignant pleural mesothelioma ***(8,9)***.

After quick preliminary attempts, the protocol yielded reproducible results demonstrating the feasibility of PRINS and immunofluorescence. In our hands, it provides an accurate technique to study chromosome anchorage on the spindle and its segregation with respect to metaphase/anaphase "checkpoints" in various physiological or pathological situations in which cells are susceptible to aneuploidization or polyploidization. For a most general point of view, PRINS has proved to be an attractive technology and an excellent alternative to FISH as a means of achieving fluorescent *in situ* DNA labeling combined with the indirect immunofluorescent tagging of proteins.

2. Materials

2.1. Cell Cultures (see Note 1)

1. KG1a is a myeloid leukemic cell ***(10)***. Cells were grown in α-minimal essential medium supplemented with penicillin (100 U/mL), streptomycine (100 µg/mL), glutamine (2 mmol/L), and 10% fetal calf serum (Gibco, BRL, Invitrogen SARL, Cergy Pontoise, France).
2. Megakaryocytes from CD34+ cells: CD34+ cells were obtained from human bone marrow of healthy patients undergoing hip surgery with their informed consent. Briefly, cells were separated over a Ficoll-metrizoate gradient (Ficoll-separating solution; Biochrom KG, Berlin, Germany); then, CD34+ cells were isolated using the immunomagnetic beads technique (Miltenyi, Biotec, France ***[11]***). The purity was usually in the range 70 to 90%. CD34+ cells were grown for 5 d in Iscove's modified Dulbeco's medium (Gibco BRL) containing penicillin (100 U/mL), streptomycine (100 µg/mL), glutamine (2 mmol/L; Sigma Chemical Co, St Louis, MO), α-monothioglycerol (76 nmol/L; Sigma), 1.5% deionized bovine serum albumin (BSA; Cohn fraction V; Sigma); 1/100 of insulin-transferrin selenium X (Gibco BRL); and sonicated lipids (20 µg/mL). The culture medium was supplemented with PEG-rhuMGDF (10 ng/mL) and recombinant human stem cell fac-

tor (SCF 50 ng/mL; generous gifts from Kirin Brewery, Tokyo, Japan, and Amgen, Thousand Oaks, CA, respectively).

2.2. Slide Preparation

1. 1X phosphate buffer saline (PBS): 1/10 solution in distilled water from PBS 10X (Gibco BRL; *see* **Note 2**).
2. Cytospin: cytospin 2 (Shandon, Eragny, France).
3. Fixative: 90% methanol/10% acetic acid (Carlo Erba, Rodano, Italy; *see* **Note 3**).
4. Cell suspension medium: 1X PBS, 0.1% ethylene diamine tetraacetic acid, 0.2% BSA.
5. Permeabilization medium: 1X PBS, 1% Triton X-100 (Sigma).
6. Denaturation solution: 70% formamide, 30% 2X standard saline citrate (SSC), pH 7.0–7.2 (*see* **Note 4**).
7. 20X SSC: 3*M* NaCl, 0.3 sodium citrate, pH 7.4 (Gibco, BRL).
8. 2X SSC: 1/10 20X SSC buffer in distilled water (*see* **Note 2**).
9. Ethanol series: 70%, 85%, 100%.

2.3. PRINS Reaction

1. Primers: Alpha-satellite primers able to discriminate between chromosomes were produced for the human ***(12,13).*** In the present study, we used the followed primers, which specific for centromeres of human chromosomes 1 and 7, respectively:
   1c: 5'ATTCCATTAGATGATGACCCCTTTCAT3'
   7c: 5'AGCGATTTGAGGACAATTGC3'
2. *Taq* polymerase and specific buffer, including $MgCl_2$ (Qbiogen, Illkirch, France).
3. 2'-Deoxyribonucleotide 5'-triphosphate (dNTPS): Ultrapure dNTPs set (each 100 m*M*; Pharmacia Biotech, St Quentin en Yveline, France).
4. Fluorescein-12-2'-deoxyuridine 5'-triphosphate (dUTP; Roche Molecular Biochemicals, Meylan, France (*see* **Table 1**).
5. Slides: 76 × 26× 1 mm (SuperFrost LLR2BN from CML, Nemours, France).
6. Thermo cyclers: we used two cyclers alternatively; the Hybaid Omnigen (Hybaid Limited, Teddington, UK) and the Crocodile III, Appligene (Qbiogen).
7. Rubber cement.
8. Tween-20 (Sigma).
9. 20X SSC (Gibco, BRL).
10. dNTPs mix (aliquoted by 10 µL and frozen): 120 µL of pure distillated water, 20 µL of each 100 m*M* dATP, dTCP, dGTP, 20 µL of a mother solution of 2.5 m*M* 2'-deoxythymidine 5'-triphosphate (dTTP; 1/40 dilution of the original 100 m*M* dTTP).
11. Washing solution: 4X SSC, Tween-20, pH 7.0–7.2 (200 mL 20X SSC, 5 mL of a 10% aqueous solution realized from Tween-20 and 795 mL of distillated water; *see* **Note 2**).

2.4. Indirect Immunofluorescence

1. First antibody: anti-β-tubulin antibody (monoclonal from mouse; Sigma; *see* **Table 1**).

Table 1
Antibodies and Fluorescent Reagents Used in the Study

Reagent	Type	Species	Dilution	Fluorochrome	Max/Abs-em	Origin
Fluorescein-12-dUTP	dNTP		1/100	FITC	492/520 nm	Boeringer
Anti-b tubulin	IgG monoclonal	Mouse	1/40[e]			Sigma
Anti-Fc mouse	F(ab)'2	Donkey	1/50[e]	TRITC	550/570 nm	Jackson immunoresearch
TOTO3	Intercalant		1/1500[e]		642/660 nm	Molecular probes
DAPI	Intercalant		1/20,000		360/460 nm	Sigma

Table 2
Observation Systems Used For Acquisition With the Confocal Microscope

Laser beam	λ	Fluorochrome	Dection filters
Blue argon	488 nm	FITC	BP 505-550
Green helium neon	543 nm	TRITC	BP 560-615
Red helium neon	633 nm	TOTO3	LP 650

2. Second antibody: tetramethylrhodamine isothiocyanate (TRITC)-labeled anti-mouse $F(ab')_2$ fragments (from donkey) (Jackson Immunoresearch, West Grove, PA; *see* **Table 1**).
3. DNA counterstained once: TOTO 3-iodide (Molecular Probes, Paris, France; *see* **Table 1**).
4. 1 m*M* Dimethyl sulfoxide (DMSO) (Sigma).
5. DNA counterstained twice: 4',6-diamino-2-phenyllindole (DAPI; Sigma; *see* **Table 1**).
6. Antifading: Vectashield anti-fading (Vector Laboratories, Burlingame, UK).

2.5. Image Acquisition

1. Fluorescence observation: Zeiss epifluorescent axioplant 2 microscope (Carl Zeiss, Germany) equipped with immersion plan-neofluar objectives, using a tri-CCD camera and Vysis computer software (Smart capture 2).
2. Refined images: LMS 510 laser scanning confocal microscope Zeiss with Plan-apochromat ×63/NA 1.4 and ×100/NA1.4 objectives (*see* **Table 2**).

3. Methods

A schematized view of the protocol is presented in **Fig. 1** (*see* **Note 5**).

3.1. Slide Preparation and Conservation

1. Harvesting of the cells and spreading: in our experiments, the cells were harvested by centrifugation at 200*g* for 10 min and suspended in cell suspension medium. Then, they were cytocentrifuged on cover slips (24 × 36 mm) for 4 min at 500*g* using a cytospin (*see* **Note 6**).
2. Immediately fix the cells in a 90% methanol/10% acetic solution during 4 min and then rinse and rehydrate for 5 min in 1X PBS (*see* **Note 3**).
3. Stockade: At this step, cells can be used or allowed to dry before frozen for stockade in hermetic boxes with a desiccator-like silica gel (*see* **Note 7**). To use some of the frozen cover slips, warm the box to 37°C for 30 min before opening. The remaining slides could be frozen again without damage. Start the experiment at the rehydratation stage.
4. Permeabilize the rehydrated slides in permeabilization medium (0.1% Triton in 1X PBS, s*ee* **Note 8**) for 7 min. Then, dehydrate in 70%, 85%, and 100% ethanol whashes (4 mn each) and allow to dry.

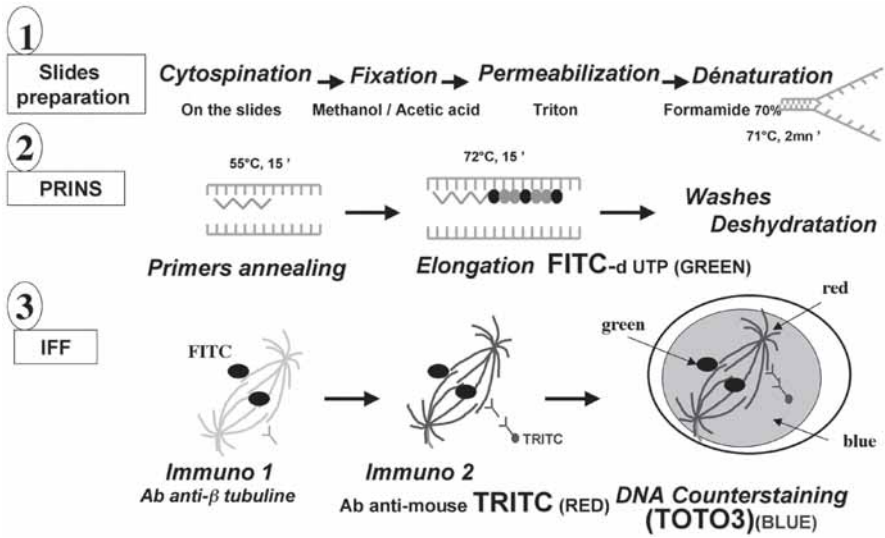

Fig. 1. The PRINS-IFF methodology.

5. Denature the slides in the denaturation solution at 71°C for 2 min and then stop them in a series of cold ethanol washes maintained at 4°C in ice (*see* **Note 9**): 70%, 80%, 90%, and 100%, 2 min each, respectively, except for the last, during which the slides are allowed to stay for 4 min. After drying the slides at room temperature, immediately perform the PRINS reaction (*see* **Note 10**).

3.2. PRINS Reaction

1. Prepare the PRINS reactional mixture ***(14)***: for a mix of 50 µL (x number of cover slips + one): 42.5 µL of distillated water, 5 µL of *Taq* polymerase buffer (with $MgCl_2$), 0.5 µL of dNTPs mix, 0.5 µL of fluorescein-12-dUTP, 1 µL of primers (100 p*M*) and 0.5 µL (2 units) of *Taq* polymerase. Maintain the mix on ice and shield it from the light (*see* **Note 11**).
2. Perform PRINS reactions on a programmable thermal cycler equipped with a flat plate block (*see* **Notes 10** and **12**). The PRINS reaction consists of two programmed steps: 15 min corresponding to the specific annealing temperature of the primers (55°C in our case), followed by 15 min at 72°C to allow the *in situ* chain elongation (*see* **Note 13**). Only one cycle is realized. Slides corresponding to the number of cover slips are put on the block and the cycle is initiated. As soon as the annealing temperature is reached, deposit 50 µL of the PRINS reactional mix on each slide. To prevent bubbles, the denatured cover slips should be placed carefully on the slides (the cells between the cover slip and the slide) and sealed using rubber cement.
3. At the end of the reaction, remove the rubber cement and the wash cover slips three times in 4X SSC washing solution. Then, dehydrate cover slips in an ethanol

series (70%, 85%, and 100%) for 3 min each, and immediately start the immunofluorescence procedure (*see* **Note 10**).

3.3. Indirect Immunofluorescence

The indirect immunofluorecence procedure is performed in the dark to prevent bleaching of the label.

1. Specific immunoreaction: Apply primary mouse anti-β-tubulin antibody (1/50 dilution in 1X PBS) for 30 min (*see* **Note 14**), followed by three quick (1 min each) washes in 1X PBS.
2. Revelation by the secondary (labeled) antibody: incubate with donkey TRITC-labeled anti-mouse $F(ab')_2$ fragments for 30 min (*see* **Note 14**) and wash three times in 1X PBS.

3.4. Counterstaining and Mounting

1. The DNA is first counterstained with 1/3000 TOTO3-iodide in 1 m*M* dimethyl sulfoxide for 30 min.
2. Wash the cover slips in 1X PBS.
3. Mount the cover slips on slides with 30 µL of Vectashield anti-fading containing DAPI (*see* **Note 15**).

3.5. Observation and Image Acquisition

1. Classical acquisitions are made using a Zeiss epifluorescent microscope equipped with tri-CCD camera and Vysis computer software (Smart capture 2). An example of simultaneous labeling obtained is illustrated in **Fig. 2** with the following specific labeling: chromosome centromeres labeled in green by PRINS, achromatic spindle in red by IIF of the β tubulin, and the whole DNA in blue using DAPI as counterstains.
2. Confocal acquisitions: Refined images are captured using a Zeiss LMS 510 laser scanning confocal microscope, either with Planapochromat ×63/NA 1.4 or ×100/NA1.4 objectives. The three laser excitations are used: 488 nm, 543 nm, and 633 nm, for FITC, TRITC, and TOTO 3, respectively (*see* **Table 2**). Serial optical sections of 0.7 mm (image collected at 0.5-mm intervals) along the *z*-axis of the cell are collected sequentially for each marker and overlaid to obtain a three-dimensional reconstruction. **Figure 3** provides an example of such labeling obtained on endomitotic megacaryocytes.

4. Notes

1. The cells lines and the culture conditions described here correspond to the set of problems that we had to solve and to our original publications (***3***,***7***), but the methodology can be easily transposed to other types of cells.
2. Solutions 1X PBS, 2X SSC, and 4X SSC-Tween, prepared from commercial concentrated solutions, can be stored several weeks at 4°C.
3, The fixative solution is always prepared extemporaneously. Notice that for a better preservation of the antigenic sites, the percent of acetic acid is reduced in

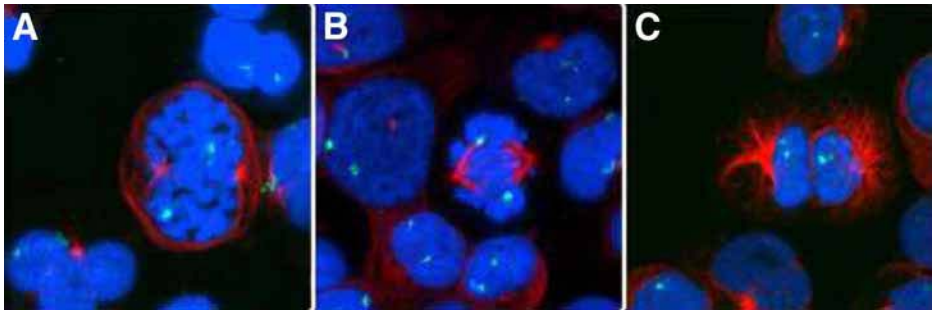

Fig. 2. Simultaneous PRINS (human chromosome 1 centromeres) and IIF (achromatic spindle) on diploid cells (KG1a): Primer 1c and fluorescein-16-dUTP were used for PRINS and the targeted centromeres are labeled in green. Microtubules were stained in red by indirect immunofluorescence with an anti-β tubulin antibody, followed by incubation with TRITC anti-mouse $F(ab')_2$ fragment. The whole DNA is counterstained in blue by DAPI. The picture acquisition was made using a Zeiss epifluorescent microscope using a tri-CCD camera and Vysis computer software (Smart capture 2). We observed two green dots, corresponding to the centomeres of the two targeted chromosomes 1, at prophase (**A**) and metaphase (**B**). Four dots, equally distributed on both sides of the cytokinetic apparatus, were present when sister-chromatids and centromeres separated from the beginning of anaphase to the onset of cytokinesis, two on each side of the equator (**C**). (Color version of this figure available in ebook.)

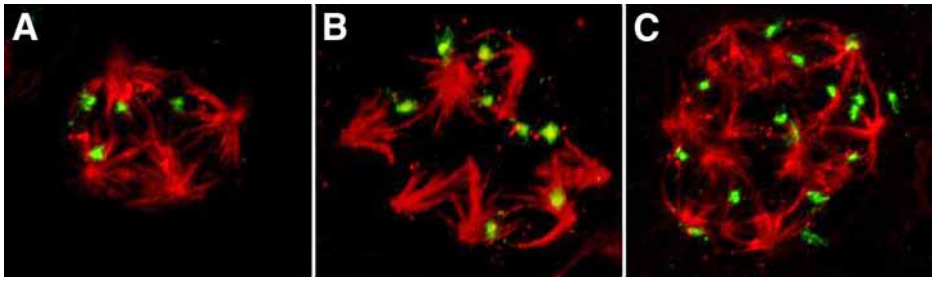

Fig. 3. Asymmetrical repartition of chromosome 1 on multipolar spindle of endomitotic megakaryocytes. Double staining obtained respectively by PRINS (green) and IIF (red) procedure in endomitosis 4n to 8n (**A**), 8n to 16n (**B**), and 16n to 32n (**C**). The blue whole DNA counterstain, evidenced by TOTO3, was not integrated on this picture. Centromeres of chromosomes 1 are asymmetrically distributed among the different spindle-poles because some of them are not associated with green dot. Images were captured using a Zeiss LMS 510 laser scanning confocal microscope Zeiss. (Color version of this figure available in ebook.)

comparison with the conventional cytogenetic fixative (25%), and the duration of the fixation itself is as short as possible.

4. For a better conservation, we store the formamide and, particularly, the opened flasks at 4°C in the dark. The 70% formamide solution used for denaturation also can be conserved at 4°C in the dark, preventing evaporation. However, remove the solution after four reaction runs or after a month.
5. We experimented using a different sequencing of the method, but we observed that the tubulin epitope was far better preserved when immunostaining was performed after DNA labeling.
6. If our cell spreading system cells don't require cover slips, it is advantageous to use slides because cover slips can be easily broken.
7. The PRINS reaction is sensitive to DNA breaks. It is important that the slides that will are frozen be perfectly dried. Opening the boxes after a 37°C incubation prevents the formation of condensation on the slides. In the same way, after closing the boxes, we let them sit for 30 min at room temperature before putting them at –20°C so that the desiccator operates well on the atmosphere of the box.
8. First prepare an 10% triton solution in distillated water.
9. It is important that pH is 7.0–7.2. In the same way, it is important that the denaturation temperature is truly 71°C and the duration of denaturation is respected. Consequently, we operate slide by slide. The first denatured slides can delay in the last ethanol bath.
10. If you are not ready to perform the PRINS reaction, you can delay it by keeping the slides in the last ethanol bath; this tactic also can be used for the immunofluorescence procedure.
11. All the PRINS reagents are stored at –20°C. We always add the *Taq* polymerase to the mix just before starting the PRINS reaction.
12. For a better efficiency, do not drive more than four slides together. If cell preparations are on slides, of course, put the slides on the block and, after putting the mix, protect with cover slips and seal with rubber cement. Protect this preparation from the light.
13. A high temperature (72°C during elongation) is used for the elongation step, implying that the studied epitope must be heat resistant. No problems were discovered in our own experiments.
14. 25 µL of antibody solution should be placed between cover slip and slide. Rubber cement is not required, but incubate at 37°C in a moist chamber. Incubations also could be made at room temperature. Don't forget to protect the preparation from light.
15. Put in suspension the original lyophilized DAPI in 1 mL of distillated water. Prepare a 1/100 solution. For each 200 µL of Vectashield, add 1 µL of this solution. We store all reagents at –20°C.

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4

PRINS for the Detection of Unique Sequences

Stephen S. Wachtel and Avirachan T. Tharapel

Summary

Primed *in situ* labeling (PRINS) can be used to localize single-copy genes and unique sequences. Using a modified PRINS method that incorporates multiple primers for the same sequence, single-step annealing and extension, anti-*Taq* DNA polymerase antibody, and stringent washing, we localized the human *SRY* gene to Yp11.31-p11.32 in chromosome preparations *in situ*. Locus-specific oligonucleotide probes (i.e., PRINS primers) were annealed to chromosomal DNA fixed on glass slides and extended in the presence of the four trinucleotide precursors, biotin-16-2'-deoxyuridine 5'-triphosphate, Tris-HCl, KCl, $MgCl_2$, bovine serum albumin, and *Taq* DNA polymerase. After reaction with avidin-conjugated fluorophores, the resulting signals could be visualized by fluorescence microscopy in metaphase spreads and in interphase nuclei. This method could prove useful for unique sequences in general.

Key Words: PRINS; chromosome; gene localization; single-copy genes; unique sequences; *SRY* gene; FISH.

1. Introduction

For nearly two decades, fluorescence *in situ* hybridization (FISH) has been a useful and significant adjunct to the cytogenetic evaluation of gene deletions and subtle chromosome rearrangements. However, in recent years, a new method, primed *in situ* labeling (PRINS) has been applied to the localization of α-satellite sequences and to the gross identification of individual chromosomes ***(1,2)***. According to the PRINS method, laboratory-synthesized oligonucleotide probes (i.e., PRINS primers) are used instead of cloned DNA for the *in situ* localization of individual genes. The PRINS primers are annealed to complementary target sequences on chromosomes and are extended in the presence of labeled nucleotides ***(3)*** utilizing *Taq* DNA polymerase. Chromosomal DNA acts as template for the extension and the labeled nucleotides as substrate for the DNA polymerase.

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Although PRINS initially was used to detect repetitive sequences, such as the α-satellite, *Alu*, and telomeric repeats ***(1,2,4)***, the method also could be used to identify a single copy sequence, the X-linked *factor IX* gene ***(5)***. That work represents a significant biological milestone because every known gene sequence is a potential primer source and, thus, potentially is amenable to fine analysis by the PRINS method.

We modified the PRINS method for increased sensitivity and applied the TSA™ Biotin System to localize the sex-determining *SRY* gene in XX men, in a woman with XY gonadal dysgenesis, and in an azoospermic man with Xp–Yp interchange. Modifications included the use of day-old slides, use of 0.02 *N* HCl to remove loosely bound protein, the application of supernumerary primers for a single target locus, single-step annealing and extension, the use of TaqStart monoclonal antibody, and stringent washing in standard saline citrate (SSC) to minimize background. Our results enabled visual confirmation of the molecular data and precise localization of the *SRY* gene in all cases ***(6)***. In other studies ***(7)***, we mapped the *SOX3* gene by using the same methods. It follows that further development of the PRINS method may allow localization of any gene or exon in chromosome preparations *in situ*, provided that the nucleotide sequence is available.

2. Materials

2.1. Lymphocyte Culture

1. Glass vacutainer tubes containing heparin (Becton Dickinson, East Rutherford, NJ).
2. RPMI-1640 culture medium (RPMI-1640) (Gibco Invitrogen, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS; Gibco Invitrogen). Stored at 4°C.
3. Gentamycin sulfate (Irvine Scientific, Santa Ana, CA). Added to RPMI-1640 at 10 mg/mL.
4. Sodium heparin (Elkins-Sinn, Cherry Hill, NJ). Stored at 4°C and added to RPMI-1640 at room temperature (1000 U/500 mL).
5. L-Glutamine (Gibco Invitrogen, Bethesda, MD). Stored at –20°C and added to RPMI-1640 at room temperature (4 mL/500 mL).
6. Phytohemagglutinin (PHA), lyophilized (Gibco Invitrogen): Reconstitute one bottle of PHA with 10 mL of distilled water. The reconstituted PHA should be stored at 4°C and prepared fresh every 30 d. It should be stored at 4°C and reconstituted at room temperature in distilled or deionized water.
7. KCl-Na citrate hypotonic solution (Fisher Scientific, Pittsburgh, PA): Add 0.560 g of KCl to 100 mL of distilled or deionized water to make a 0.075 *M* solution. Add 0.8 g of Na citrate to 100 mL of distilled or deionized water to make 0.8% Na citrate solution. Mix the two solutions just before use: 2:1, KCl:Na citrate. Store at room temperature and prepare a fresh mixture every 7 d.
8. Fixative solution: 3:1 methanol:glacial acetic acid.

2.2. Collection of Cells and Preparation of Slides

1. Colcemid (Gibco Invitrogen). Purchased in lyophilized form, stored at 4°C and used at room temperature after reconstitution in distilled water. Discard after 1 mo.
2. Methanol (Fischer Scientific).
3. Glacial acetic acid (Fischer Scientific).
4. Glass slides and cover slips (Fisher Scientific).

2.3. *Primed* In Situ *Hybridization*

1. Programmable thermal cycler equipped with a flat plate for holding slides (MISHA, Shandon Lipshaw, Pittsburgh, PA; *see* **Note 1**).
2. Dimethylsulfoxide (DMSO; Sigma Genosys, St. Louis, MO) molecular biology or high-performance liquid chromatography-grade.
3. Ethanol (Fischer Scientific).
4. Oligonucleotide primers (Research Genetics, Huntsville AL; *see* **Note 2**). The following probes for the *SRY* gene were high-performance liquid chromatography-purified, and stored at –20°C:
   5'-GCAGGGCAAGTAGTCAACGTT-3'
   5'-AAGCGACCCATGAACGCATTC-3'
   5'-AGAAGTGAGCCTGCCTATGTT-3'
   5'-GCCGACTACCCAGATTATGGA-3'
5. Trinucleotides (e.g., dATP, etc.): TSA kit; Tyramide Signal Amplification, for chromogenic and fluorescence *in situ* hybridization and immunohistochemistry (NEN Life Science Products, Boston, MA).
6. Biotin-16-2'-deoxyuridine 5'-triphosphate (dUTP; Roche Molecular Systems, Alameda, CA; formerly Boehringer-Mannheim).
7. Tris-HCl buffer (Sigma, St. Louis, MO).
8. Magnesium chloride ($MgCl_2$) (Fischer Scientific).
9. Potassium chloride (KCl; Fisher Scientific).
10. Bovine serum albumin (Invitrogen Life Technologies, Carlsbad, CA).
11. *Taq* DNA polymerase (Amplitaq, Perkin-Elmer, Foster City, CA) with TaqStart antibody (Clontech Laboratories, Palo Alto, CA).
12. 20X SSC: 3 *M* NaCl, 0.3 *M* Na citrate, pH 7.0 (Invitrogen Life Technologies).
13. Formamide-SSC (Invitrogen Life Technologies).
14. TSA Biotin System. The kit should be kept at 4°C until used, although the blocking reagent may be kept at room temperature. With proper storage, the kit is useful for 6 mo, after which the contents may become unstable.
    a. Prepare biotinyl tyramide stock solution: reconstitute biotinyl tyramide (amplifcation reagent) by addition of 0.3 to 1.2 mL of DMSO (the amount of DMSO will depend on the particular NEN kit used: 0.3 mL for 50–150 slides; 1.2 mL for 200–600 slides). Because DMSO freezes at 4°C, it is necessary to thaw the stock solution after removal from refrigerator.
    b. Prepare TNT washing buffer: 0.1 *M* Tris-HCl, pH 7.5, 0.15 *M* NaCl, 0.05% Tween-20. The manufacturer advises that PBS may be used as an alternative buffer and that 0.3% Triton X-100 may be used instead of 0.05% Tween-20.

c. Prepare TNB blocking buffer: 0.1 *M* Tris-HCl, pH 7.5, 0.15 *M* NaCl, 0.5% Blocking Reagent (in kit). According to the manufacturer's protocol, the blocking reagent should be added slowly to buffer in small amounts while stirring. The mixture should be heated to 60°C with continuous stirring *(see* **Note 3**). The blocking reagent should be dissolved completely (this may take several hours). After preparation, store at –20°C.

15. Tween-20 (Sigma Genosys).
16. Triton X-100 (Sigma Genosys).

2.4. Microscopy, Visualization, and Scoring

1. Streptavidin-fluorophore conjugate (FITC or Texas red, also known as SpectrumRed; Vysis Inc. Downers Grove, IL).
2. Counterstain: 4',6-diamino-phenylindole (2 × 500 µL, DAPI II) (Vysis Inc.).
3. Microscope equipped for fluorescence microscopy. We used an Olympus B-Max, U-M510 with triple bandpass filters, Red, Green, and DAPI Bandpass, and Triple Bandpass Filter Set, DAPI/Green/Red (Vysis Inc.).
4. We used the Color Capturing System (Applied Imaging, Santa Clara, CA).
5. For scoring, 20 metaphase spreads (and 80 interphase nuclei) were analyzed for each locus in each subject.

3. Methods

For these tests, approx 5 mL of whole venous blood is drawn into heparinized syringes or glass vacutainer tubes. The methods for lymphocyte culture in advance of PRINS are detailed below.

3.1. Preparing the Culture Medium

1. Mix 100 mL of fetal bovine serum with 400 mL of RPMI-1640 in a separate (500-mL) bottle. Agitate well. Prepare fresh mixture every 30 d. Store at 4°C.
2. To each 600 mL of medium, add 3 mL of sodium heparin, 2 mL of gentamycin sulfate, and 4 mL of L-glutamine.

3.2. Initiation of Lymphocyte Culture

1. Two cultures are initiated for each subject.
2. For each culture, add 10 mL of culture medium to two 15-mL centrifuge tubes.
3. Using a Pasteur pipet, inoculate each centrifuge tube with 12 to 15 drops of whole blood. Each centrifuge tube represents a separate lymphocyte culture. Label the tubes.
4. Add 0.15 mL of PHA to each tube and mix well.
5. Incubate the cultures at 37°C for 72 h (in general, the cultures may remain undisturbed for the full 3-d period, although some groups gently agitate the cultures a few times each day).

3.3. Harvesting the Lymphocytes

1. Using an 18-gage syringe needle, add five drops of reconstituted colcemid (10 μg/mL) to each culture.
2. After mixing well, incubate the cultures for 1–2 h at 37°C.
3. Centrifuge at 250*g* for 10 min.
4. Remove the supernatant and break up the pellet by agitation with a vortex mixer.
5. Add 10 mL of hypotonic KCl-Na citrate mixture to each culture. Suspend the cells by gently inverting the centrifuge tubes manually. Allow the tubes to stand at room temperature for 30 min.
6. Gently suspend the cells again by inverting the tubes. Then, add 2 mL of freshly prepared methanol–glacial acetic acid fixative directly to the contents of each tube. Mix the contents by inverting the tubes, and centrifuge the tubes again at 250*g* for 10 min.
7. Remove all of the supernatant from each culture. Gently "thump" the tubes to break up the cellular pellets.
8. Add 10 mL of fresh fixative to each culture. Resuspend the cell pellets by inverting the tubes and allow the cultures to stand at room temperature for 10 min, and centrifuge.
9. Repeat **step 8**.
10. The next steps are performed in a "harvesting room" with a humidifier. For maximal chromosome spreading, adjust the humidity to 55–65%.
11. Set a hot plate to 65°C and check the temperature with a surface thermometer.
12. Centrifuge the cell suspension at 250*g* for 10 min.
13. Remove the supernatant from the centrifuge tubes and add fixative to the pellet drop by drop until the suspension becomes "semiclear." The amount of fixative required will depend on the size of the pellet. The final cell concentration may have to be adjusted after evaluation of the first slide.

3.4. Preparing Slides for PRINS

High-quality chromosome preparations are a key factor for obtaining satisfactory results after hybridization on glass slides (*see* **Note 4**).

1. Clean microscope slides are placed in a slide tray containing deionized water. The slides are chilled in a refrigerator.
2. One cold slide at a time is removed from the slide tray and maintained in a slanting position on a stand or a device (at approximately a 10- to 20-degree angle).
3. Drop or place 40 to 50 μL of cell suspension on the slide. Allow the cell suspension to roll down the slide. Wipe the back of each slide and shake off excess fixative and water. Label slides and place on a hot plate at 65°C for 2–3 min.
4. Store slides at room temperature for 24 h, after which they are ready for the labeling steps (*see* **Note 5**).

3.5. *Primed* In Situ *Labeling: Standard Protocol*

1. Immerse slides in 0.02 *N* HCl for 20 min (*see* **Note 6**).
2. Immerse slides in 70% formamide-SSC, pH 7.0 for 2 min at 72°C, to denature chromosomal DNA.
3. Dehydrate slides by passage through a cold ethanol series, 70%, 85%, and 100% EtOH, 5 min each. Air-dry.
4. Prepare reaction mixture in a final volume of 40 µL containing 50 pmol of each oligonucleotide primer (*see* **Note 7**); 0.2 m*M* each dATP, dCTP, dGTP; 0.02 m*M* dTTP; 0.02 m*M* biotin-16-dUTP; 50 m*M* KCl; 10 m*M* Tris-HCl, pH 9.0; 2 m*M* $MgCl_2$; 0.01% BSA; and 1 unit *Taq* DNA polymerase with TaqStart antibody (*see* **Note 8**). Pipet 40 µL of reaction mixture onto freshly prepared slide (*see* **Note 9**).
5. Cover the working area of the slide completely with a glass cover slip. Attach the ends of the cover slip in place with a thin application of rubber cement.
6. Incubate slides. Our incubations are carried out on a programmable thermal cycler equipped with a flat plate for slides (MISHA, Shandon Lipshaw) *(see* **Note 1**). The program consists of one cycle of 10 min at annealing temperature (55–75°C) with an additional 30 min at 72°C for extension (*see* **Note 10** for computation of annealing temperature).
7. After extension, slides are removed from cycler, the cover slips are removed, and the slides washed in 0.4X SSC at 72°C for 2 min to stop the reaction (*see* **Note 11**).
8. In our studies, biotin-labeled nucleotides are detected with the TSA Biotin System.

3.6. *Signal Amplification Using the TSA Biotin System*

1. For each test, dilute the stock solution of biotinyl tyramide 1:50 with 1X amplification diluent to prepare the working solution; 100 to 300 µL of working solution are needed for each slide.
2. After hybridization, block slides by incubation with 100 to 300 µL of TNB buffer. This may be performed for 30 min in the humidified chamber of the cyler with biotin-labeled probes: 100 to 300 µL of SA-HRP (streptavidin-horseradish peroxidase from the TSA kit) diluted 1:100 in TNB buffer. Fluorescein-labeled probes may be substituted: 100 to 300 µL of anti-fluorescein-HRP (NEN) diluted 1:250 in TNB buffer. Optimal concentrations of HRP-labeled reagents should be determined for individual laboratories.
3. Wash slides in TNT buffer with agitation three times for 5 min at room temperature.
4. Pipet 100 to 300 µL of the working solution onto each slide. Maintain slides at room temperature for 5 to 10 min.
5. Repeat washing **step 3**.

3.7. *Fluorescence Microscopy and Visualization*

1. To each slide, add 100–300 µL of streptavidin–fluorophore conjugate diluted in TNB buffer; use dilution recommended by manufacturer: streptavidin–Texas red (NEN) is used at 1:500.

2. Place slides in a humidified chamber for 30 min at room temperature.
3. Wash slides in TNT buffer, with agitation, three times for 5 min at room temperature.
4. For staining of chromosomes (counterstain) add two drops of DAPI II and mount for microscopy. Blot excess DAPI, cover, seal the ends of the cover slip with rubber cement, and refrigerate at 4°C for 30–60 min (*see* **Note 12**).

4. Notes

1. An alternative programmable cycler, the "HYBrite Denaturation/Hybridization System," is produced by Vysis Inc. However, annealing and extension can be accomplished on thermoblocks or even in metal containers suspended in hot water baths ***(3).*** As always with PRINS, temperature is critical and must be carefully controlled.
2. In our experience, signal is increased by use of multiple (as many as four or five) primers for a single locus and by single-step annealing and extension.
3. Innis and Gelfand ***(8)*** note that at 20°C, TRIS buffer has a pK_a of 8.3, and $\Delta\ pK_a$ of –0.021/°C. Therefore, the actual pH of Tris-HCl may vary during thermal cycling.
4. An unamplified slide without TSAG reagents and an amplified slide without primer should be included as controls for each hybridization.
5. Slides should be kept moist during the PRINS procedure. If a humidified chamber is not available, cover slides with a damp paper towel in a closed box. If a humidifier is available, maintain humidity at 55–65% for optimal chromosome spreading (**Subheading 3.3.**, **step 10**).
6. Treatment of slides with 0.02 *N* HCl removes loosely bound protein, thereby rendering DNA more accessible to the primers.
7. For each study, the primer concentration should be optimized. New England Nuclear recommends a 10-fold reduction in "probe" (primer) concentration as optimal. This step is a critically important one in PRINS because an improper concentration of probe can obviate development of the hybridization signal.
8. TaqStart monoclonal antibody binds *Taq* DNA polymerase, thereby minimizing nonspecific amplification and formation of primer dimers
9. Reagents should completely cover cells or metaphase spreads on slides.
10. After counting the A, C, G, and T nucleotide residues of the primers, compute annealing temperatures by use of either of the following formulas:

$$T_M = 69.3 + 0.41\ (\%\ G + C) - 650 / L$$

where L = the length of the primer = the total number of nucleotides in the primer.

$$T_M = 4\ (G + C) + 2\ (A + T)$$

When different temperatures are obtained, the results may be averaged. In general, satisfactory annealing occurs at temperatures between 55 and 75°C. Higher temperatures increase annealing specificity.

11. Background staining is minimized by stringent washing of slides in SSC.
12. Low signal may be corrected by titration of HRP conjugate to optimize concentration, by increasing incubation time or concentration of amplification reagent,

or by addition of a step to optimize penetration of reagents. Background staining may be minimized by decreasing concentration of HRP conjugate or primers, by increasing endogenous peroxide quenching, by filtration of buffers, or by increasing number of washes or the length of washes.

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5

Single-Copy and Point Mutation Analysis (Rolling-Circle PRINS)

Jørn Koch

Summary

This protocol is a prototype for a series of upcoming procedures aimed at the detection of single molecules of nucleic acids *in situ*. It uses circular probes that, upon hybridization to their target molecule, can template rolling-circle DNA synthesis, if the target also provides a primer for initiation of the DNA synthesis. This primer may be endogenous or artificial, for example, obtained through cleavage with a restriction enzyme. In this format, the primed *in situ* labeling (PRINS) product takes the form of a long-tandem repeat copy of the probe, covalently attached to the site of synthesis, and tagged it with copies of the probe. The amplification is sufficient for the detection of single, oligonucleotide size, targets and, depending on the probe format, the probe may detect variations in the targets down to single base substitutions.

Key Words: PRINS; RCA; rolling circle.

1. Introduction

Organisms are made of tissues, and tissues are made of cells. Any tissue is composed of a mixture of many types of cells. On a genetic level, these cells differ in their gene expression and, in cancers, also in their DNA. Because the performance of any tissue and, ultimately, the organism, depends on the behavior of its constituent cells, a thorough understanding of biological processes in biology and in pathology requires studies of the individual cells and the biomolecules within the cells. The dominant molecular biology methods do not provide this insight because they rely on the analysis of biomolecules isolated from extracts of pools of cells. To get the full picture, it is necessary to obtain data on the individual cells in a format that also gives spatial information on the biomolecules inside the cells and the cells inside the tissues. In essence, this means that cells and biomolecules should be studied *in situ*, which

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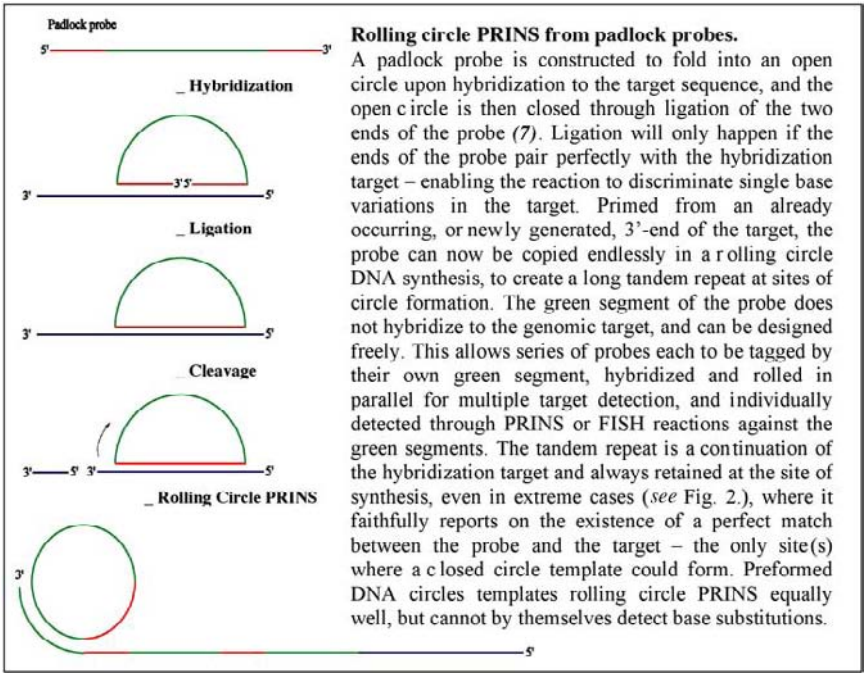
has been possible since the introduction of *in situ* hybridization and immunohistochemistry. Unfortunately, any of the two techniques has a limited sensitivity (cannot see ultimately low amounts of targets) and resolution (cannot see ultimately small features of the targets). I therefore undertook the development of a new technology with the prospect of providing single-molecule DNA and RNA detection *in situ* at single-nucleotide resolution approx 20 yr ago ***(1–4)***. The underlying principles of the new technology were that short oligonucleotides were used as probes for the target definition and DNA synthesis at sites binding the probes was used for target visualization. With oligonucleotide probes being sensitive down to single base variations in the target sequence, it should be possible to identify point mutations (or single-nucleotide polymorphisms) in target sequences and, with the DNA synthesis being capable of proceeding for tens of kilobases in vitro, it should be possible to generate enough DNA from a single priming event for it to become visible in the microscope. This potential has now been turned into a series of protocols, as evidenced by this book. In most protocols, a number of priming events per target are required to make it visible above background because of the low and variable frequency of priming events from endogenous 3'-ends of DNA, either generated during the life of the cell or as a consequence of the cell preparation. Thus, whereas repeated targets enables multiple priming events from a single probe, standard detection of single copy genes has required the use of a cocktail of probes for that gene ***(5,6)***. However, there are two ways in which to obtain probe-specific *in situ* DNA synthesis in the absence of endogenous DNA synthesis, one of which is the rolling-circle PRINS (**Fig. 1**) described here. The other is the dideoxy-PRINS (*see* Chapter 8).

2. Materials

2.1. Basic Ingredients and Instrumentation

1. Slides containing ethanol-fixed cells (or standard chromosome spreads).
2. 37°C humidified incubator.
3. Cover slips of appropriate size (e.g., 24 × 60 mm to completely cover a standard slide).
4. Coplin jars or other suitable containers for washing the slides.
5. 10X phosphate-buffered saline (PBS) and/or 20X standard saline citrate (SSC; PBS and SSC may to some extent replace each other in the buffers used).
6. Restriction enzyme and (10X) buffer (e.g., Roche).
7. Bovine serum albumin (BSA), DNase free.
8. Glycerol (*see* **Note 1**).
9. Formamide (fresh or deionized).
10. Padlock probe(s).
11. T4 DNA ligase and 10X buffer (enzyme and buffer are supplied together by Roche).



Rolling circle PRINS from padlock probes. A padlock probe is constructed to fold into an open circle upon hybridization to the target sequence, and the open circle is then closed through ligation of the two ends of the probe *(7)*. Ligation will only happen if the ends of the probe pair perfectly with the hybridization target – enabling the reaction to discriminate single base variations in the target. Primed from an already occurring, or newly generated, 3'-end of the target, the probe can now be copied endlessly in a rolling circle DNA synthesis, to create a long tandem repeat at sites of circle formation. The green segment of the probe does not hybridize to the genomic target, and can be designed freely. This allows series of probes each to be tagged by their own green segment, hybridized and rolled in parallel for multiple target detection, and individually detected through PRINS or FISH reactions against the green segments. The tandem repeat is a continuation of the hybridization target and always retained at the site of synthesis, even in extreme cases (*see* Fig. 2.), where it faithfully reports on the existence of a perfect match between the probe and the target – the only site(s) where a closed circle template could form. Preformed DNA circles templates rolling circle PRINS equally well, but cannot by themselves detect base substitutions.

Fig. 1. Rolling-circle PRINS from padlock probes. (Please *see* color insert following p. 48.)

12. φ-29 DNA polymerase and 10X buffer (enzyme and buffer are supplied together by Fermentas).
13. dATP, dCTP, dGTP dTTP: 100 m*M* (Lithium salts) from Roche.
14. 20% Tween-20.
15. Identifier oligonucleotides.
16. Fluorochrome labeled anti-digoxigenin for detection of digoxigenin-labeled DNA (Fab fragment, Roche). Fluorochrome-labeled Streptavidin for detection of biotin-labeled DNA (Roche, Vector Laboratories); optional.
17. Antifade mounting medium (Vectashield from Vector Laboratories or *p*-phenylenediamine from Sigma) containing either of the following:
18. 0.4 µ*M* DAPI (Sigma; for blue counterstaining of DNA) or
19. 0.5 µg/mL Propidium iodide (Sigma) for red counterstaining of DNA.
20. Fluorescence microscope with standard excitation and emission filters (e.g., 81000 filter set from Chroma Technology).

2.2. Mixtures

1. Ethanol series: 70%, 90%, 99% (v/v). Can be stored at room temperature, but sucks water from the air (changes concentration).

2. 0.1% (w/v) Pepsin in 0.1 *M* HCl: freshly prepared and preheated to 37°C (it may take several minutes to dissolve pepsin even at 37°C); optional.
3. RNase A mixture (optional): for 100 µL, mix 80 µL of H_2O, 10 µL of 1 µg/µL RNase A, and 10 µL of PBS. Prepare fresh.
4. Restriction enzyme mixture (Roche): for 100 µL, mix 78 µL of H_2O, 10 µL of 1 µg/µL BSA, 10 µL of 10X restriction buffer, and 2 µL of restriction enzyme (2 U/100 µL have proven sufficient for nick translation *in situ* ***(8)***, but we have often used more here—as much as 50U/100 µL mix). Prepare fresh.
5. λ-Exonuclease mixture (New England Biolabs): for 100 µL, mix 68 µL of H_2O, 10 µL of 10X incubation buffer, 10 µL of 1 µg/µL BSA, 10 µL of concentrated glycerol, and 2 µL (10–20 U) λ-exonuclease. Prepare fresh.
6. Denhardt's solution (4X): 400 mg of Ficoll 400, 400 mg of polyvinylpyrrolidone (PVP), 400 mg of BSA (fraction V), and H_2O to 500 mL. Filter sterilize and store at –20°C in 25-mL aliquots.
7. Hybridization mixture: for 100 µL, mix 12 µL of H_2O, 25 µL of 4X SSC, 25 µL of 40% (v/v) formamide, 25 µL of 4X Denhardt's solution, 12 µL of 2 µg/µL salmon sperm DNA, and 1 µL of each relevant DNA probe (from 1 µ*M* stock solutions). Prepare fresh.
8. Padlock removal solution: 2X SSC and 30% (v/v) deionized formamide.
9. T4 DNA ligase mixture, for 100 µL, mix 78 µL of H_2O, 10µL of 1µg/µL BSA, 10 µL of 10X ligase buffer, and 2 µL of (10 U) T4-DNA ligase. Prepare fresh.
10. Reaction mixture for rolling-circle PRINS: for 100 µL, mix 56 µL of H_2O, 10 µL of 10X polymerase buffer, 10 µL of 1 µg/µL BSA, 2 µL of 50m*M* DTT, 10 µL of concentrated glycerol, 10 µL of 10X dNTP (1X dNTP: 250 n*M* of each), 2 µL of φ-29 DNA polymerase. Prepare fresh.
11. Wash buffer: 4X SSC, pH 7.0, 0.05% (v/v) Tween-20. Store up to 1 yr at room temperature.
12. Blocking solution (optional): Dissolve 5% (w/v) nonfat dry milk in wash buffer. Centrifuge for 2 min in a microcentrifuge at maximum speed and use supernatant. This solution can be stored at –20°C for years. When in use, it is preferable to store the blocking solution at 4°C and not use it for more than 1 wk (the milk turns sour with time, and the same may happen to the milk powder if it sucks water from the air).

3. Methods

The following protocol largely corresponds to the recently published procedure for the genotyping of individual mitochondrial genomes *in situ* ***(2)*** and was derived in collaboration with Mats Nilsson and Ulf Landegren at Uppsala University. It should be pointed out that it is an experimental protocol, and some considerations should be made before applying it:

- The detection efficiency for a given target is, for now, 1–10%. We are working to increase it, but for the time being, there is this limiting factor to consider.
- The protocol is for detection on standard chromosome slides. Preliminary results suggest that enhanced reactions can be obtained in some sort of well format. This

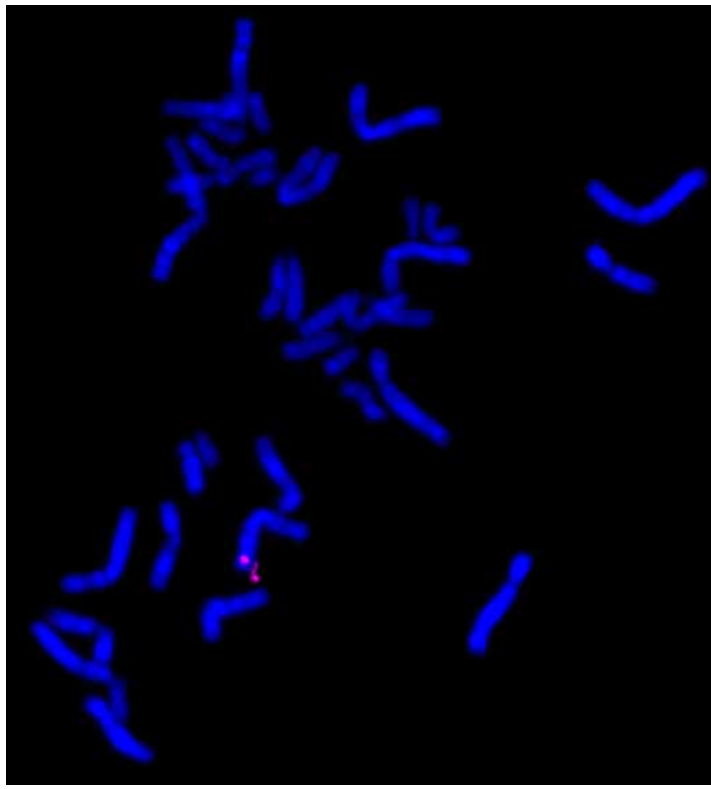


Fig. 2. Detection of a gene in the long arm of human chromosome 6. The efficiency of the reaction is not 100%, so only one chromosome 6 presents with a signal. This signal consists of a well-defined spot on one chromatid and a long thread of DNA looping out from the other chromatid. This long thread of DNA was the product of a reaction where the rolling-circle PRINS proceeded for an extended period of time, and the rolling circle at some point wandered out of the chromatin. However, even in this extreme case, it is clear at which locus the signal originated. (Please *see* color insert following p. 48.)

would be in agreement with findings in other *in situ* reactions that consume large amounts of reagents locally, such as the cycling PRINS ***(9)***.

- All possible steps have been included. We have not been able to document an effect of the RNase treatment, the pepsin may not be relevant for chromosome spreads (**Fig. 2**), preformed circles do not require ligation *in situ*, and at least some of the dehydrations may be replaced by equilibration in the subsequent reaction buffer. Furthermore, detection of endogenous 3'-ends does not require a restriction digest. Thus, although the full procedure can be completed within a single working day, it may still be possible to cut it down for individual applications.

1. Decide how large a region of the slide is to be used and choose a cover slip and a volume of reaction mixture that fits the area. In general use, 1 µL of mixture for each millimeter in cover slip length. To cover a standard slide completely, prepare 60 µL of each mixture and cover with a 24 × 60-mm cover slip. To cover a smaller area, prepare less mixture (and use a smaller cover slip).
2. Rinse the slide in a fairly neutral, low-salt buffer without excessive buffering capacity (e.g., PBS or SSC).
3. Put slide into preheated pepsin solution and incubate 1 min at 37°C. The duration of this step is critical. Try to avoid over- or underdigestion of samples.
4. Rinse the slide in the same buffer used in **step 2**. Dehydrate the specimen by immersing the slide successively in an ethanol series (3 min each in 70%, 90%, and 99% ethanol). Drain off the ethanol and air-dry the slide. The reaction can be paused after this and all subsequent dehydrations.
5. Incubate the slide with the RNase A mixture under the cover slip for 30 min at 37°C in a humidified incubator.
6. Rinse, dehydrate, and air-dry the slide as in **step 4**.
7. Digest the target DNA on the slide and under cover slip with a suitable restriction enzyme so that the hybridization target will be in the vicinity of a free 3'-end by incubating with a restriction enzyme mixture under cover slip for 30 min at 37°C (or other temperature suitable for the particular enzyme).
8. Rinse, dehydrate, and air-dry the slide as in **step 4**.
9. Remove the target complementary DNA strand by incubating with λ-exonuclease mixture under cover slip for 30 min at 37°C. This step provides an enzymatic "denaturation" of the genomic DNA, where one strand of the DNA is removed to leave the other as a single-stranded DNA, ready for hybridization of the probe. Standard denaturation procedures have also been useed ***(1)*** but, for the time being, the enzymatic denaturation is our favorite.
10. Rinse, dehydrate, and air-dry the slide as in **step 4**.
11. Hybridize the padlock probe(s) at a concentration of 10 to 200 nm (e.g., 100 n*M*) in hybridization mixture under cover slip for 30 min at 37°C.
12. Rinse away excess probe by incubating 5 min each in two changes of wash buffer at 37°C, then dehydrate and air-dry the slide as described in **step 4**.
13. Incubate the slide with DNA ligase mixture under cover slip for 30 min at 37°C.
14. Remove padlock probes not turned into closed circles by washing 15 min each in two changes of padlock removal solution at 42°C. Rinse, dehydrate, and air-dry slide as in **step 4**.
15. Incubate the slide with the rolling-circle PRINS reaction mixture under cover slip for 30 min at 37°C.
16. Rinse, dehydrate, and air-dry the slide as in **step 4**.
17. Incubate with identifier oligonucleotides at a concentration of 10 to 250 nm in hybridization mixture for 30 min at 37°C. The identifier oligonucleotides are used for the identification of the rolling-circle product from a given padlock probe (which may be applied as a padlock probe within a mixture of padlock probes that are all hybridized and rolled in parallel; *see* **Notes 2** and **3**).

18. Rinse away excess probe by incubating in two changes of wash buffer for 5 min each at 37°C.
19. Counterstain and mount slides. For blue counterstaining of DNA, mount slide in 20 µL antifade mounting medium containing 0.4 µ*M* of DAPI. For red counterstaining of DNA, Mount slide in 20 µL of antifade mounting medium containing 0.5 µg/mL propidium iodide.
    Examine slides by fluorescence microscopy using standard excitation and emission filters.

4. Notes

1. Glycerol prevents evaporation and enhances exonuclease digestion and polymerization in concentrations of 5–20%. Such concentrations may be reached without the addition of extra glycerol if, for example, the nucleotides and the relevant buffers are stored in 50% glycerol. Storing key reagents in 50% glycerol has the advantage that they can be pipetted directly from –20°C, thus avoiding freezing and thawing. The activity of the restriction enzyme also can be enhanced with glycerol, but this enhancement may not be desirable, as it takes the form of star activity, with the enzyme cutting at relaxed specificity.
2. The criterion for the selection of these identifiers is that they should specifically recognize a given rolling-circle product—any oligonucleotide from 16-mers and up that does not recognize genomic sequences should be suited for the purpose.
3. Because it is possible to co-hybridize and co-roll multiple padlock probes on the same specimen, it is preferable to generate a class of unique identifiers that can also be co-hybridized for the identification of the individual rolling-circle products *in situ*. Each identifier is synthesized with one fluorophore attached to it, and the fluorophores are chosen among the fluorophores usually used for FISH. Instead of using labeled oligonucleotides and FISH to identify the rolling-circle products, the identifier oligonucleotides may also be used as PRINS primers, e.g. in a DNA cascade reaction ***(1)***. However, the FISH procedure is the best tested at this point and the approach best suited for multiplexing. For PRINS detection, it may be preferable to design the backbone from only three nucleotides so it can be detected by dideoxy PRINS.

Acknowledgments

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6

Analysis of Sperm Aneuploidy by PRINS

Franck Pellestor, Brigitte Andréo, Jacques Puechberty, Geneviève Lefort, and Pierre Sarda

Summary

Based on the direct *in situ* mixing of the colors of different fluorochromes (fluorescein isothiocyanate, tetramethylrhodamine isothiocyanate, Cascade Blue) incorporated in sequential primed *in situ* labeling (PRINS) reactions, a new multicolor PRINS procedure is described, allowing the rapid and distinct *in situ* labeling of three or four human chromosomes. Each PRINS reaction consists of a unique 5-min step for annealing and elongation. In combination with the 0.5 *M* NaOH pretreatment for simultaneous *in situ* denaturation and decondensation of sperm nuclei, this technique has been adapted to human sperm nuclei for the direct assessment of aneuploidy.

Key Words: PRINS; multicolor; human sperm; NaOH decondensation; chromosomes; aneuploidy.

1. Introduction

Aneuploidy is the most common class of chromosome abnormalities in humans, accounting for a significant proportion of miscarriages and multiple congenital malformation syndromes in liveborns. Direct chromosomal analysis of spermatozoa constitutes an essential approach for the investigation of the occurrence and etiology of chromosomal abnormalities in humans under a wide variety of clinical conditions. The advent of molecular genetic techniques has brought forth new procedures for *in situ* chromosomal analysis and has opened the way for comprehensive studies on the occurrence of aneuploidy in human spermatozoa. To date, numerous chromosomal analyses on human sperm have been performed using fluorescence *in situ* hybridization (FISH ***[1,2]***). These reports have demonstrated the efficiency of the *in situ* labeling procedure on male gametes but also have pointed out the limitations of FISH on this biological material, which essentially are linked to the size of the probes and the reliability of the associated sperm decondensation treatments ***(3,4)***.

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The primed *in situ* (PRINS) reaction offers an efficient alternative approach for the direct chromosome analysis of human spermatozoa. Based on the use of chromosome-specific primers, the PRINS method combines the high sensitivity of the polymerase chain reaction with the cytological localization of DNA sequences. Since its introduction ***(5)***, the PRINS protocol has been continuously optimized, and several studies have demonstrated that PRINS labeling was more effective and exhibited higher specificity than FISH on human sperm ***(6–8)***.

In the conventional PRINS procedure, the identification of several chromosomes is performed by *in situ* sequential labeling of each targeted chromosome by using chromosome-specific primers and different reporter molecules or fluorochromes. This initial multicolor PRINS procedure was adapted successfully to human spermatozoa, and has been shown to be very well adapted to this material because of the small size of the PRINS primers and the requirement of a moderate sperm nucleus decondensation ***(9)***. However, a blocking step based on the use of ddNTP is used between each labeling reaction to avoid the mixing of the labeling. This intermediate reaction significantly extends the total duration of the multi-PRINS procedure and may lead to a prejudicial decrease in the intensity of the labeling of the first targeted chromosomes.

Recently, Yan et al. ***(10)*** have reported a new multicolor PRINS protocol based only on *in situ* mixing of two fluorochromes for generating the distinct and specific labeling of three chromosomes. Because this sequential procedure without blocking steps significantly simplifies the multicolor PRINS protocol and improves the efficiency of PRINS labeling, we have adapted this new method on human sperm samples. Improvements in the timing and the procedure have been introduced, leading to the elaboration of a new ultra-rapid three- or four-color PRINS protocol for sperm chromosomal screening, described in this chapter. The principles of this new PRINS labeling procedure are illustrated in **Fig. 1**.

2. Materials

2.1. Human Sperm Preparation

1. Phosphate-buffered saline (PBS; Gibco BRL, Eragny, France).
2. 99% Methanol (Prolabo, Paris, France).
3. Ethanol series: 70%, 90%, 100% (Prolabo).
4. Glacial acetic acid (Prolabo).
5. 0.5 *M* NaOH (Merck Eurolab, Nogent-sur-Marne, France).
6. 20X Standard saline citrate (SSC): 3 *M* NaCl, 0.3 *M* trisodium citrate, pH 7.5 (this solution can be stored for several months at room temperature).
7. Washing buffer: 2X SSC diluted from 20X SSC.

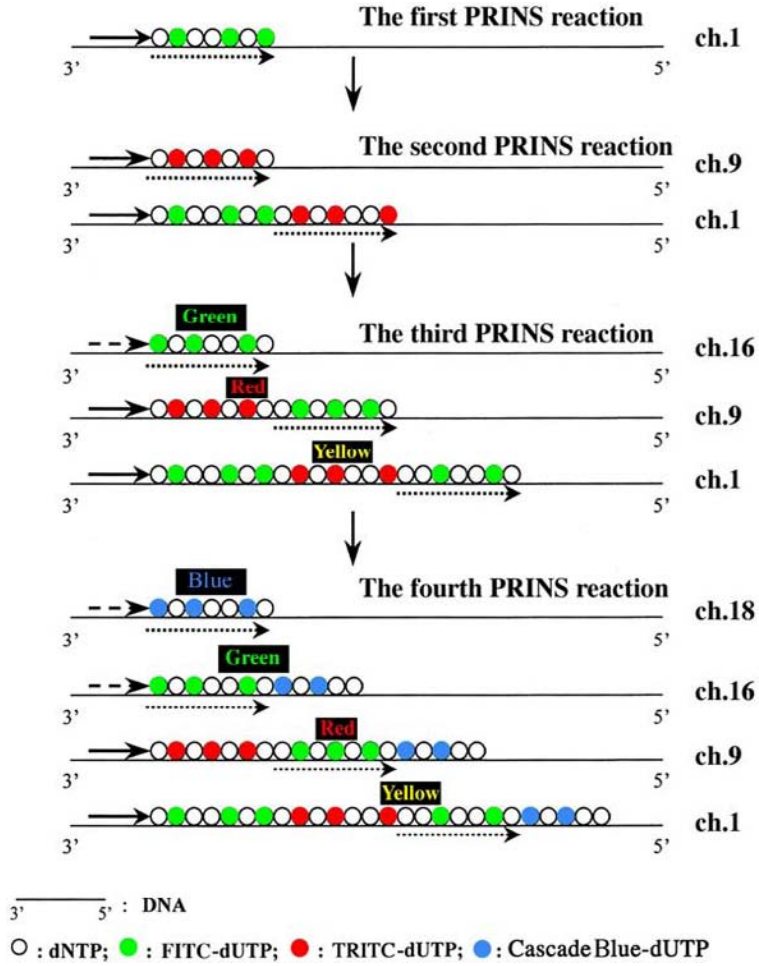

Fig. 1. Principles of the multicolor PRINS reaction (modified from Yan ***[10]***). Four sequential PRINS reactions, using specific centromeric primers for four chromosomes and three distinct fluorochrome-dUTPs (FITC-11-dUTP, TRITC-6-dUTP, Cascade Blue-7-dUTP) are performed without intermediate 3'-end blocking steps. The *in situ* mixing of the colors led to the final generation of four distinct colored spots (green, red, yellow, and blue), allowing the specific identification of each targeted chromosome. In this illustration, the chromosome 1 is successively labeled by the *in situ* incorporation of FITC, TRITC, FITC, and Cascade Blue, leading to a final yellow color. Chromosome 9 is labeled by FITC, TRIC, and Cascade Blue, leading to a red color. Chromosome 16 is labeled by the incorporation of FITC and Cascade Blue, resulting in a green color. Finally, chromosome 18 only incorporates Cascade Blue dUTP, giving a blue color. (Please *see* color insert following p. 48.)

8. Twin-frost glass microscope slides (CML, Nemours, France). The slides must be cleaned by soaking in absolute ethanol to which concentrated HCl has been added at the rate of 1 mL/100 mL. The slides are removed from the acid/alcohol and polished with a clean piece of muslin just before dropping the sperm suspension.
9. Light microscope Leica DMLB with ×10, ×40 magnification (Leica France, Rueil-Malmaison, France).

2.2. Multicolor PRINS

1. dATP: 100 m*M* solution (Roche Diagnostics, Meylan, France) diluted 1:10 with sterile distilled H_2O.
2. dCTP: 100 m*M* solution (Roche Diagnostics) diluted 1:10 with sterile distilled H_2O.
3. dGTP: 100 m*M* solution (Roche Diagnostics) diluted 1:10 with sterile distilled H_2O.
4. dTTP: 100 m*M* solution (Roche Diagnostics) diluted 1:100 with sterile distilled H_2O.
5. 1 m*M* Fluorescein isothiocyanate-12-dUTP (FITC-12-dUTP) (Roche Diagnostics).
6. 1 m*M* Tetramethylrhodamine isothiocyanate-6-dUTP (TRITC-6-dUTP) (Roche Diagnostics).
7. Cascade Blue-7-dUTP 1m *M* (Molecular Probes, Leiden, The Netherlands).
8. 1X PBS.
9. Bovine serum albumin (BSA; Sigma, St. Louis, MO).
10. *Taq* DNA polymerase (Roche Diagnostics) or Ampli*Taq* (Perkin Elmer, Foster City, CA).
11. 10X *Taq* buffer: 500 m*M* KCl, 100 m*M* Tris-HCl, pH 8.3, 15 m*M* $MgCl_2$.
12. Oligonucleotide primers at 50 pmol/µL (*see* **Table 1** and **Note 1**).
13. Sterile distilled water.
14. Tween-20 (Roche Diagnostics).
15. Washing buffer (diluted from 20X SSC): 4X SSC, 0.05% Tween-20.
16. 1.5-mL Sterile microcentrifuge tubes (Eppendorf AG, Hamburg, Germany).
17. Cover slips (22 × 32 mm; CML).
18. Coplin jar (50 mL).
19. Programmable thermal cycler equipped with a flat pate block (Hybaid Ltd., Teddington, UK).

2.3. Detection and Microscopy

1. 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI; Sigma).
2. Propidium iodide (Sigma).
3. Antifade solution Vectashield (Vector Labs, Burlingame, CA).
4. Cover slips (20 × 40 mm; CML).
5. Rubber cement (Artos, Strasbourg, France).
6. Epifluorescence Microscope Leica DMRB (Leica France) equipped with ×40 and ×100 Plan FluoTar objectives, and with a DAPI single band-pass filter (Leitz filter A, cat. no. 513804), a FITC single band-pass filter (filter I3, cat. no. 513808), a TRITC single band-pass filter (filter N2.1, cat. no. 513812), a FITC/TRITC double band-pass filter (filter G/R, cat. no. 513803) and a triple filter

Table 1
Characteristics of the Principal PRINS Primers Used in Human Cytogenetics

Name	Locus	Chromosome	Sequence	Annealing temp. (°C)	Optimal conc. (pmpl)
Alu S	alu	All	5'-GCCACTGCACTCCAGCCTGGG-3'	55	150
Alu R	alu	All	5'-GCCTCCCAACGTGCTGGGATTACAG-3'	55	150
Cons	αsat	All	5'-TCTTTTTGTAGAATCTGCAAGTGGATA-3'	54	150
J52	sat III	1	5'-ATTCCATTAGATGATGACCCCTTTCAT-3'	61	100
1c	αsat	1	5'-ATTCCATTAGATGATGACCCCTTTCAT-3'	61	100
2c	αsat	2	5'-CTGTTCAACACTGRG-3'	71	150
3c	αsat	3	5'-TGAGTTGAACACACACGTAC-3'	66	150
5c	αsat	5	5'-TTCTGTCTAGCCTTACAGGAAAA-3'	70	150
7c	αsat	7	5'-AGCGATTTGAGGACAATTGC-3'	56	100
8c	αsat	8	5'-CTATCAATAGAAATGTTCAGCACAGTT-3'	67	150
9c	sat III	9	5'-AATCAACCCGAGTGCAATC-3'	56	150
10c	αsat	10	5'-ACTGGAACGCACAGATGACAAAGC-3'	63	200
11c	αsat	11	5'-GAGGGGTTTCAGAGCTGCT-3'	65	200
12c	αsat	12	5'-GTTCAAATTCACAGAGTAT-3'	60	200
12A	αsat	13	5'-TGATGTGTGTACCCAGCT-3'	60	100
16c	αsat	16	5'-TTCTTTTCATACCGCATTCT-3'	53	75
17c	αsat	17	5'-AATTTCAGCTGACTAAACA-3'	51	200
18c	αsat	18	5'-ATGTGTGTCCTCAACTAAAG-3'	65	100
21A	αsat	21	5'-TGATGTGTGTACCCAGCC-3'	61	150
Xc	αsat	X	5'-GTTCAGCTCTGTGAGTGAAA-3'	68	75
D600	sat III	Y	5'-TCCATTCGATTCCATTTTTTTCGAGAA-3'	56	100

(filter B/G/R, cat. no. 513836) for simultaneous observation of DAPI/Cascade-Blue, FITC, and TRITC signals.
7. For image capturing, we use the software Metasystem Isis, Version 5.0 (Metasystem, Altlusshein, Germany).

3. Methods

3.1. Preparation of Sperm Slide

1. Freshly ejaculated sperm sample is allowed to liquefy at room temperature for 30 min.
2. Dilute 1 mL of sperm in 1X PBS (1:10) and centrifuge for 5 min at 500*g*.
3. Remove the supernatant and resuspend the pellet in 10 mL of 1X PBS for a new wash by centrifugation (5 min at 500*g*).
4. Resuspend the pellet in 1 mL of fresh fixative (3:1 methanol:glacial acetic acid).
5. Fix for 1 h at –20°C.
6. Using a Pasteur pipet, drop one droplet of the sperm suspension on a clean microscope slide from a height of approx 10 cm.
7. The slide is air-dried and checked under the light microscope (×10 and ×40) to ensure that both the cell concentration and the spreading are optimum (*see* **Note 2**). Using a diamond marker, draw a circle on the underside of the slide to mark where the spermatozoa are.
8. Store the slide for 2 d at room temperature, preferentially in a hermetic box in order to avoid dust deposits (*see* **Note 3**).
9. Immediately before the PRINS reaction, the slide is denatured in 0.5 *M* NaOH at room temperature for 4 min (*see* **Note 4**).
10. Wash the slide twice for 2 min at room temperature in 2X SSC.
11. Pass the slide through an ethanol series (70%, 90%, and 100%), 2 min each step, and air-dry.

3.2. Multicolor PRINS

In the three-color PRINS procedure, three sequential PRINS reactions are performed, each labeling one specific chromosome. The following labeling order is used (*see* **Note 5**):

1. FITC for the first targeted chromosome.
2. TRITC for the second targeted chromosome.
3. FITC for the third targeted chromosome.

In the four-color procedure, this labeling order is supplemented with a fourth PRINS reaction using Cascade Blue (*see* **Fig. 1**).

3.2.1. Three-Color PRINS Procedure

1. Prepare a reaction mixture in a final volume of 50 µL containing: 0.2 m*M* dATP, dCTP, and dGTP, 0.02 m*M* dTTP, 0.02 m*M* FITC-12-dUTP, 50 m*M* KCl, 10 m*M* Tris-HCl, pH 8.3, 1.5 m*M* $MgCl_2$, 0.01% BSA, 200 pmol of oligonucleotide primer; and 2.5U of *Taq* DNA polymerase. In practice, mix in a sterile microcen-

trifuge tube: 1 µL of each 1:10 diluted dATP, dCTP, and dGTP; 1 µL of the 1:100 diluted dTTP; 1 µL of FITC-12- dUTP; 1 µL of BSA; 5 µL of 10X *Taq* buffer; 0.5 µL of the *Taq* DNA polymerase; 4 µL of the specific primer (for instance, the primer specific for chromosome 1 according to the procedure illustrated in **Fig. 1**); and distilled water to 50 µL.

2. Place the reaction mixture under a 22 × 32 cover slip on the denatured slide, and transfer to the heating block of the thermal cycler.
3. Set up the PRINS program and start the reaction. The program consists of a unique 5-min step at the specific annealing temperature of the primer involved for both *in situ* annealing and elongation (*see* **Note 6**).
4. While this first reaction is running, prepare the reaction mixture for the second PRINS reaction as described previously but instead incorporate the specific primer for the second targeted chromosome (for instance, chromosome 9 according to **Fig. 1**), and TRITC-6-dUTP.
5. On completion of the program, carefully remove the cover slip from the slide.
6. Wash the slide twice for 2 min at room temperature in 1X PBS.
7. After draining the excess 1X PBS off the slide, and before the slide is completely dry, put the second PRINS reaction mixture on the slide, and cover with a 22 × 32 cover slip.
8. Place the side again on the plate of the thermal cycler.
9. Set up the program for the second PRINS reaction: 5 min at the annealing temperature specific to the second primer used.
10. Start the program.

No additional denaturation is required after the first PRINS reaction because the sperm DNA remains denatured through the PRINS incubations.

11. While this second reaction is running, prepare the reaction mixture for the third PRINS reaction, incorporating the specific primer for the third targeted chromosome (for instance, chromosome 16 according to **Fig. 1**) and FITC-12-dUTP.
12. At the end of the second reaction, remove the cover slip from the slide and repeat the washing, **steps 6** and **7**.
13. Before the slide is completely dry, put the third PRINS reaction mixture on the slide, and cover with a 22 × 32 cover slip.
14. Place the slide on the thermal cycler.
15. Set up the program for the third PRINS reaction: 5 min at the annealing temperature specific to the third primer used.
16. Start the program.
17. At the end of this third reaction, the slide is transferred to 4X SSC, 0.05% Tween-20 for two washes (3 min each) at room temperature, with gentle agitation.

3.2.2. Four-Color PRINS Procedure

In the four-color procedure, the third reaction is followed by a fourth reaction with the primer specific for the fourth targeted chromosome (for instance, chromosome 18 as indicated in **Fig. 1**). No additional denaturation is needed.

1. Prepare the reaction mixture for the fourth PRINS reaction, incorporating the specific primer for the fourth targeted chromosome and Cascade Blue-7-dUTP.
2. At the end of the reaction, remove the cover slip from the slide.
3. Transfer the slide in 4X SSC, 0.05% Tween-20 for two washes (3 min each) at room temperature, with gentle agitation.

3.3. Detection and Microscopy

1. Drain the excess washing solution off the slide.
2. Mount the slide in Vectashield antifade solution containing either DAPI (0.3 µL/mL) or a mix of propidium iodide (0.3 µL/mL) and DAPI (0.3 µL/mL).
3. Cover with a 22 × 40 cover slip and seal the cover slip with rubber cement.
4. Examine the slide under the epifluorescence microscope, preferentially using first the triple or double band-pass filter, and confirming the coloration of the fluorescent spot with single band-pass filters. **Figure 2** shows some typical results obtained on sperm nuclei.
5. For aneuploidy estimate in sperm samples, strict scoring criteria must be used (*see* **Note 7**).
6. The slide may be stored in the dark at 4°C for several months.

4. Notes

1. Human chromosome-specific primers are oligonucleotides, typically 18 to 35 bases long, specific for α-satellite DNA sequences. They are determined in the α-satellite DNA sequences of each chromosome. Several specific primers can be defined for the same chromosome. The concentration of the appropriate primer must be experimentally determined. Usually 200 pmol per slide in 50-µL reaction mixture is optimal.
2. The spreading of spermatozoa on the slide must be homogeneous to facilitate the screening of the preparation and to limit incorrect scoring when performing chromosomal analysis on sperm nuclei. It is particularly important to avoid both aggregation and excessive dilution of cells on the slide. A density of 70–100 spermatozoa per field under a ×40 objective is optimal. Consequently, the washing procedure is a critical step. It must be performed taking into account the quality of the initial sperm sample, that is, the viscosity after liquefaction, the sperm concentration, the agglutination of spermatozoa, and the presence of cellular debris or various cell types (leukocytes, epithelial cells, immature germinal cells, bacteria). In the case of low-quality sperm samples, one or two additional washes by centrifugation may be required to obtain adequate smeared sperm slides.
3. The age of the slides is critical for the success of the PRINS reaction. In our experience, the best results are obtained with 2-d-old spreads. Sperm suspensions can also be stored in fixative at –20°C for several months, and fresh slides made by centrifuging to collect a pellet, resuspending the pellet in fresh fixative, and dropping the suspension on clean slides, which will be aged 2 d at room temperature. Using slides that are older than 1 wk can be successful, but leads to reduced labeling sensitivity.

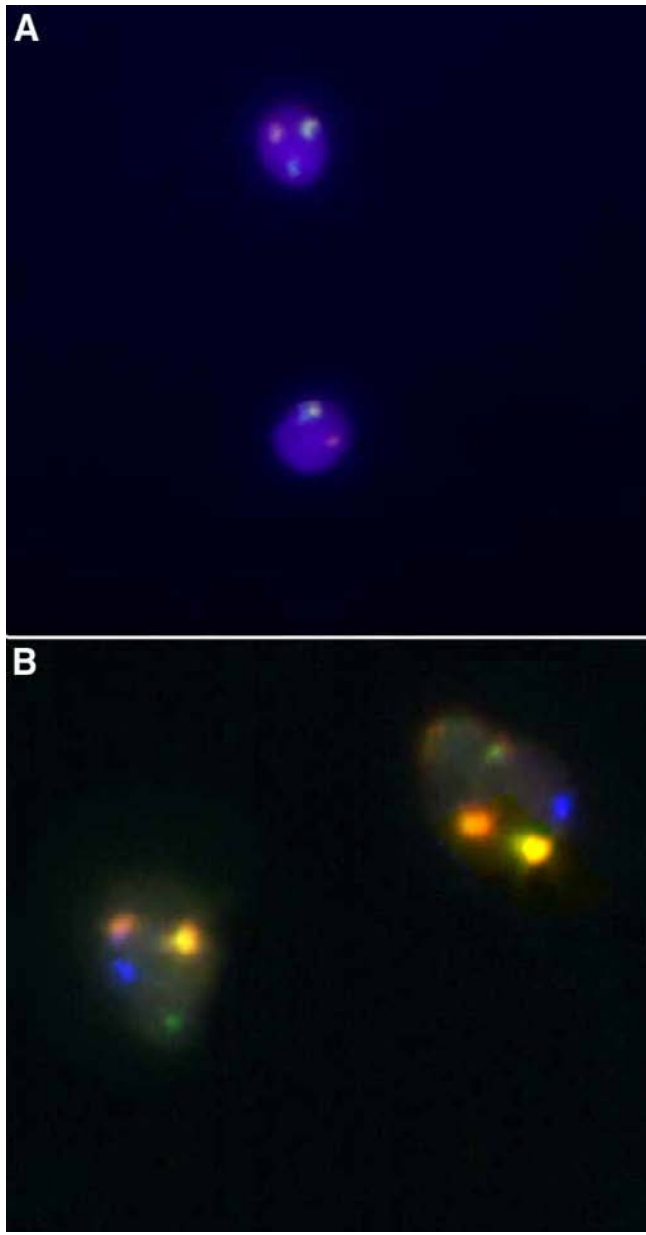

Fig. 2. Examples of *in situ* PRINS labeling of human sperm nuclei. (**A**) Direct three-color PRINS labeling on chromosome 1 (yellow), chromosome 9 (red), and chromosome 7 (green) using the FITC/TRITC/FITC labeling order. (**B**) Direct four-color PRINS labeling on chromosome 1 (yellow), chromosome 9 (red), chromosome 16 (green), and chromosome 18 (blue) using the labeling order FITC/TRITC/FITC/Cascade Blue. (Please *see* color insert following p. 48.)

4. The use of NaOH solution allows the simultaneous denaturation and decondensation of sperm nuclei, with the possibility of a rapid control of the degree of nucleus decondensation under the microscope. The success of the technique depends to a high degree on the quality and efficiency of the decondensing protocol. Initially, we used 3 *M* NaOH, but numerous experiments and a comparison of the results in terms of quality of the preparation obtained, led us to adopt a 0.5 *M* NaOH solution. Combined with PRINS, this method provides homogeneous sperm decondensation, and subsequently a high level of sperm *in situ* labeling. The duration of 0.5 *M* NaOH treatment depends on the age of the sperm preparation slides. The longer the slides are aged, the longer they need 0.5 *M* NaOH treatment: 2-d-old, 4 min; 3-d-old, 5 min; 4-d-old, 6 min. The NaOH treatment induces uniform swelling of the sperm nucleus 1.5 to 2 times its normal size, and maintains the characteristics and shape of the sperm nucleus, including the tail, allowing one to differentiate between spermatozoa and other cells, such as leukocytes or immature germ cells, present in the ejaculate.
5. For the three-color PRINS reactions, the combination order FITC/TRITC/FITC gives the best results, with distinct red, green, and yellow spots. When using the reversed labeling combination TRITC/FITC/TRITC, there is no pure yellow color; instead, a mixed orange color is obtained for the first labeled chromosome. Indeed, a mean ratio of green to red color of 70%:30% must be respected to obtain well-defined green, red, and yellow signals. In the four-color procedure, the addition of the blue label does not affect the final coloring of the three previously labeled targets. The Cascade Blue dye provides a color that contrasts well with the longer-wavelength green and red fluorophores.
6. In the conventional PRINS protocol, the labeling reaction consists of two programmed steps corresponding to (1) the annealing of the primer to the DNA target sequence and (2) the primer elongation catalyzed by the *Taq* DNA polymerase. Because the range for optimal activity of *Taq* DNA polymerases is between 55 and 75°C, we decided to use a unique one-temperature step for both annealing and elongation, which allows us to simplify the protocol and shorten the duration of the reaction. The optimal primer annealing temperature is determined empirically, and slight adjustments may be necessary according to the thermal cycler used. The conditions described here must be taken as indications only, and not as definitive values.
7. Strict scoring criteria are required to ensure accurate aneuploidy estimates on human sperm samples. Sperm nuclei are scored as haploid when they display distinct signals in different colors corresponding to each labeled chromosome. Nuclei are considered disomic when they show two signals of the same color, are comparable in size and intensity, and are separated by the diameter of at least one fluorescent spot. Overlapped spermatozoa or sperm heads without a well-defined boundary should not be counted. In human sperm, the frequency of aneuploidy for a given chromosome can be extremely low. Consequently, a large number of spermatozoa must be scored to ensure that reliable disomy estimates are obtained. The statistical pitfalls associated with scoring small numbers of cells are obvi-

ous. There is a high risk of error in estimates if fewer than 2000 spermatozoa are scored. In this case, scoring one or two more or less cells can significantly change the frequency of disomy. Consequently, a minimum of 5000 spermatozoa per individual and per chromosome must be scored.

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7

In Situ PCR Protocols

Omar Bagasra and Twaina Harris

Summary

This chapter provides detailed methods and material descriptions of *in situ* polymerase chain reaction protocols. It describes all the essential components of the technique, various protocols suitable for different kinds of tissues and cell preparations, and also gives various instructions for troubleshooting. We have declined to devote detailed discussions on *in situ* hybridization because there are several excellent articles and books available on this subject.

Key Words: *In situ* hybridization; PCR; RT-PCR; probes; primers; amplification; chambers.

1. Introduction

Before one can perform *in situ* polymerase chain reactions (PCRs) using the protocols described herein, utmost care first should be taken to preserve the DNA and mRNA molecules inside the cellular structures. Therefore, we recommend using ultraclean glass slides. The slides can be purchased from any reliable source. Furthermore, it would be wise to autoclave or sterilize the slides (also discussed herein) before applying cells or tissue sections to them. We realize that in many circumstances this may not be possible because often imes a bench scientist will receive tissues slides already mounted in a clinical laboratory elsewhere.

2. Materials

1. Clean glass slides (*see* **Note 1**).
2. Slide frames or self-sealing solution.
3. Coplin jars and glass staining dishes.
4. 2% Paraformaldehyde: add 12 g of paraformaldehyde (Merck ultra pure Art no. 4005) to 600 mL of 1X phosphate-buffered saline (PBS) and heat at 65°C for

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10 min. When the solution starts to clear, add four drops of 10 *N* NaOH and stir. Adjust to neutral pH and cool to room temperature. Filter on Whatman's no. 1.

5. 10X PBS stock solution, pH 7.2 to 7.4: dissolve 20.5 g of $NaH_2PO_4 \cdot H_2O$ and 179.9 g of $Na_2HPO \cdot 7H_2O$ (or 95.5 g $Na_2 HPO_4$) in approx 4 L of double-distilled water, then adjust to the required pH (7.27.4). Add 701.3 g of NaCl and make up to a total volume of 8 L.
6. 1X PBS: dilute the 10X PBS stock at a 1:10 ratio (i.e., 100 mL of 10X PBS and 900 mL of water for 1 L). Final concentration of buffer should be 0.01 *M* phosphate and 0.15 *M* NaCl.
7. 0.3% Hydrogen peroxide (H_2O_2) in PBS: dilute stock to 30% hydrogen peroxide (H_2O_2) at a 1:100 ratio in 1X PBS for a final concentration of 0.3% H_2O_2.
8. Proteinase K: dissolve powder from Sigma in water to obtain 1 mg/mL concentration. Aliquot and store at –20°C. Prepare a working solution (6 µg/mL by diluting 1 mL of stock (1 mg/mL) into 150 mL of 1X PBS.
9. 20X standard saline citrate (SSC): dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL of water. Adjust the pH to 7.0 with a few drops of 10 *N* NaOH. Adjust the volume to 1 L with water. Sterilize by autoclaving.
10. 2X SSC: dilute 100 mL of 20X SSC in 900 mL of water.
11. RPMI Medium 1640: per 100 mL, supplement with 15 mL of fetal bovine serum, 1.5 of HEPES buffer, and 0.1 mL of gentamycin (0.1 heparin is optional).
12. Velban: reconstitute vial with 10 mL of sterile H_2O. From this solution, dilute 0.1 mL into 50 mL distilled H_2O. Store in the refrigerator.
13. EGTA hypotonic solution: dissolve 0.2 g of EGTA powder, 3.0 g of KCl, and 4.8 g of HEPES buffer into 1000 mL of distilled H_2O. Adjust pH to 7.4. Store in refrigerator and, prior to use, prewarm to 37°C.
14. Phytohemagglutinin (PHA-C): reconstitute with 5 mL of sterile H_2O. Aliquot into five 1-mL insulin syringes. Freeze four for later use and leave one in refrigerator.
15. Fixative solution: one part glacial acetic acid in three parts methanol; store at –20°C.
16. Streptavidin peroxidase: dissolve powder from Sigma in PBS to make a stock of 1 mg/mL. Just before use, dilute stock solution in sterile PBS at a 1:30 ratio.
17. Color solution: dissolve one tablet of 3-amino-9-ethyl-carbazole (AEC; Sigma) in 2.5 mL of *N*,*N*,dimethyl formamide. Store at 4°C in the dark. To prepare working solution, mix the following:

a. 50 m*M* acetate buffer, pH 5.8	5 mL
b. AEC solution	250 µL
c. 30% H_2O_2	25 µL

Make fresh before each use, keeping solution in the dark.

18. 50 m*M* acetate buffer pH 5.0: add 74 mL of 0.2 *N* acetic acid (11.55 mL glacial acid/L and 176 mL of 0.2 *M* sodium acetate (27.2 g sodium acetate trihydrate in 1 L) to 1 L of deionized water and mix.
19. *In situ* hybridization buffer (for 5 mL): 2.5 mL formamide, 500 µL of salmon sperm DNA (ssDNA; 10 mg/mL), 500 µL of 20X SSC, 1 mL of 50X Denhardt's

solution, 50 µL of 10% sodium dodecyl sulfate (SDS), and 450 µL of water. Heat to denature ssDNA at 94°C for 10 min before adding to the solution.

20. P20 Micropipet.
21. Xylene solution (EM grade, benzene free).
22. 2% AES solution: 5 mL of 3-aminopropyltriethoxysilane (AES: Sigma A-3648), and 250 mL of acetone.
23. Ethanol series: 100%, 90%, 80%, 70%, and 50% (EM grade).
24. Tissue-Tek OCT (an embedding media for frozen tissue specimens made by Miles Laboratory of Elkhart, IN).
25. RNase-free, DNase solution: 40 m*M* Tris-HCl, pH 7.4, 6 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, and 1 U/µL final volume of DNase (use RNase-free DNase, such as 10 U/µL RQ1 DNase, cat no. 776785 from Boehringer or any other reliable source).
26. 10X Reverse transcriptase (RT) reaction buffer: 100 m*M* Tris, pH 8.3; 500 m*M* KCl; 15 m*M* $MgCl_2$.
27. RT reaction solution (if using Moloney Murine Leukemia Virus [MuLV] RT enzyme, or other equivalent) for a total volume of 20.0 µL:
    a. 10X Reaction buffer 2.0 µL
    b. 10 m*M* dATP 2.0 µL
    c. 10 m*M* dCTP 2.0 µL
    d. 10 m*M* dGTP 2.0 µL
    e. 10 m*M* dTTP 2.0 µL
    f. RNasin (40 µ/µL) 0.5 µL
    g. 20 µ*M* downstream primer 1.0 µL
    h. AMVRT 20 U/µL 0.5 µL
    i. Diethylpyrocarbonate (DEPC) water 8.0 µL
28. Mixture for one-step RT-amplification (for 100 µL):
    a. 100 µ*M* Forward primer 0.5 µL
    b. 100 µ*M* Reverse primer 0.5 µL
    c. 3 m*M* Nucleotide mix (dNTP) 6.0 µL.
    d. 10 m*M* $MnCl_2$ 2.0 µL
    e. 25 m*M* $MgCl_2$ 10.0 µL
    f. 10X Transcription buffer 2.0 µL
    g. 10X Chelating buffer 8.0 µL
    h. 1.7 mg/mL Bovine serum albumin (BSA) 10.0 µL
    i. 2.5 U/mL rTth enzyme 2.0 µL
    j. DEPC-treated water 59.0 µL
29. 10X Transcription buffer: 100 m*M* Tris-HCl, pH 8.3; 900 m*M* KCl.
30. 10X Chelating buffer: 100 m*M* Tris-HCl, pH 8.3; 1 *M* KCl; 7.5 m*M* EGTA, 0.5% Tween-20; 50% (v/v) glycerol.
31. Streptavidin–alkaline phosphatase conjugate (40 µg/mL stock).
32. Blocking solution for alkaline phosphatase-based color development: 50 mg/mL BSA (protein) in 100 m*M* Tris-HCl, pH 7.8, 150 m*M* NaCl, 0.2 mg/mL sodium azide.
33. Conjugate dilution buffer for alkaline phosphatase-based color development: 100 m*M* Tris-HCl, 150 m*M* $MgCl_2$, 10 mg/mL BSA, 0.2 mg/mL sodium azide.

34. Buffer A for alkaline phosphatase-based color development: 100 m*M* Tris-HCl, pH 7.5; 150 m*M* NaCl.
35. Alkaline substrate buffer for alkaline phosphatase-based color development: 100 m*M* Tris-HCl, pH 9.5, 150 m*M* NaCl, 50 m*M* $MgCl_2$.
36. Nitro-blue-tetrazolium (NBT): 75 mg/mL NBT in 70% (v/v) dimethlyformamide, freshly prepared.
37. 4-Bromo-5-chloro-3-indolylphosphate (BCIP): 50 mg/mL in 100% dimethylformamide, freshly prepared.
38. Water-based mounting medium such as CrystalMount™ or GelMount™ or an organic solvent-based medium such as Permount™ (Fisher Scientific).
39. Light microscope (×400).
40. Ultraviolet (UV) microscope with appropriate filter range.

3. Methods

3.1. AES Silanization: Putting on the Positive Charge

1. Prepare the 2% AES solution just before use.
2. Put solution into a Coplin jar or glass staining dish and dip glass slides in 2% AES for 60 s.
3. Dip slides five times into a different vessel filled with 1000 mL of distilled water.
4. Repeat **step 3** three times, changing the water each time.
5. Air-dry in laminar-flow hood from a few hours to overnight and then store slides in a sealed container at room temperature. Try to use slides within 15 d of silanation; 250 mL of AES solution is sufficient to treat 200 glass slides.

3.2. Preparation of Tissue

3.2.1. Cell Suspensions

1. Isolate cells on a Ficoll-Hypaque density gradient (tissue-culture cells or other single-cell suspensions can also be used).
2. Wash cells with 1X PBS twice.
3. Resuspend cells in PBS at 2×10^6 or 5×10^5 cells/mL.
4. Add 10 µL of cell suspension to each well of the slide using a P20 micropipet.
5. Air-dry slide in a laminar-flow hood.

3.2.2. Cells Cultured on Slide

The slides must be sterilized after silanization and before innoculation with the culture.

1. Soak the slides in ethanol for 30 min.
2. Cell adhesion substances (i.e., fibronectin, etc) can be added on the slide surface and air-dried.
3. Place the cells on the slides and culture overnight in a sterile, humidified box (*see* **Note 2**).

3.2.3. Paraffin-Fixed Tissue

1. Place tissue sections on properly prepared slide, as described previously (*see* **Note 3**).
2. Place tissue section upon the glass surface of the slide.
3. Incubate the slides in an oven at 80°C for 1 h to melt the paraffin.
4. Dip the slides in xylene for 5 min, then in 100%, 90%, 80%, 70%, and 50% ethanol, and then in H_2O for 5 min each.
5. Dry the slides in air for 1 h.

3.2.4. Freezing the Tissue

Three methods of tissue freezing are described.

3.2.4.1. Tissue Freezing Method A

1. Cut a 1 × 1 cm piece of styrofoam from a sheet approx 3-mm thick (or use a disposable styrofoam coffee cup if a sheet is not available).
2. Cut a slice of tissue about the same size as the styrofoam square but somewhat thicker (up to 1 cm thick).
3. Pour approx 2 mL of Tissue-Tek OCT onto the styrofoam substrate.
4. Lay on the tissue.
5. Pour another 2 mL of OCT on top so that the tissue is covered with OCT.
6. Fashion an immersion tool from wire or a coat hanger so that the styrofoam/tissue sample can rest on a loop of wire with a wire handle that allows immersion.
7. Slowly lower the apparatus and sample into the liquid nitrogen; the tissue should freeze in less than 30 s.
8. Load the tissue into a cryocassette.
9. Cut or put into a –70°C freezer for storage.

3.2.4.2. Tissue Freezing Method B

1. Place the tissue into a small plastic bag designed for immersion into liquid nitrogen. These bags are common items in pathology laboratories.
2. Immerse the tissue in the liquid nitrogen.
3. Remove and either load into a cryocassette for cutting or put into a –70°C freezer for storage.

3.2.4.3. Tissue Freezing Method C

1. If liquid nitrogen is not available, tissue prepared with styrofoam and OCT (as in method A) can be wrapped in aluminum foil.
2. Place on dry ice for 10 to 15 min before storing in the deep freezer. However, some ice crystallization in the tissue may occur with this method.
3. Under no circumstance attempt to freeze the tissue by merely placing it into a –70°C freezer. This will result in an abundance of ice crystals, and the sections will not be suitable for *in situ* procedures (*see* **Note 4**).

3.2.5. Sectioning the Tissue

1. It is necessary to use as thin a section as possible, that is, as small as 6 µL in thickness.
2. Once the tissue is properly sliced (*see* **Note 5**), apply to slide.
3. Dehydrate for 10 min in 100% methanol.
4. Air-dry in a laminar flow hood.
5. Proceed to the heat-treatment step (i.e., **Subheading 3.3.1.**).

3.2.6. Archival Tissue

1. Remove the cover slip and mounting by soaking in 100% methanol (or carefully in acetone if methanol does not pry the cover slips open).
2. Deparafinnize as described previously in **Subheading 3.2.3.**
3. Because the tissue is already on a slide, one does not have a choice about the area of the slide covered by the section or what type of surface preparation to use.
4. Instead, just develop the chamber with the Frame Chamber, as shown in **Fig. 1**.
5. Load the reagents into the wells of the chamber with PCR cocktail and seal them with the plastic sealing cover paper provided.
6. Not all of the archival tissue will be subject to the amplification–hybridization procedure, but usually there is a sufficient area in the well region to get adequate results.

3.3. Slide Treatments

3.3.1. Heat Treatment

1. Place the slides with adhered tissue on a heat-block at 105°C for 5 to 120 s to stabilize the cells or tissues (*see* **Note 6**).

3.3.2. Fixation and Washes

1. Place the slides in a solution of 4% paraformaldehyde in PBS for 4 h at room temperature. Using the recommended Coplin jars or staining dishes facilitates these steps.
2. Wash the slides once with 3X PBS for 10 min, agitating periodically with an up and down motion.
3. Wash the slides with 1X PBS for 10 min, agitating periodically with an up and down motion. Repeat twice with fresh 1X PBS.
4. At this point, slides with adhered tissue can be stored at –70°C until use. Before storage, dehydrate with 100% ethanol.
5. If biotinylated probes or peroxidase-based color developments are to be used, the samples should further be treated with a 0.3% solution of hydrogen peroxide in PBS.
6. Incubate the slides overnight—either at 37°C or at room temperature, then, wash the slides once with PBS.
7. If other detection systems are to be used, proceed directly to the proteinase K digestion.

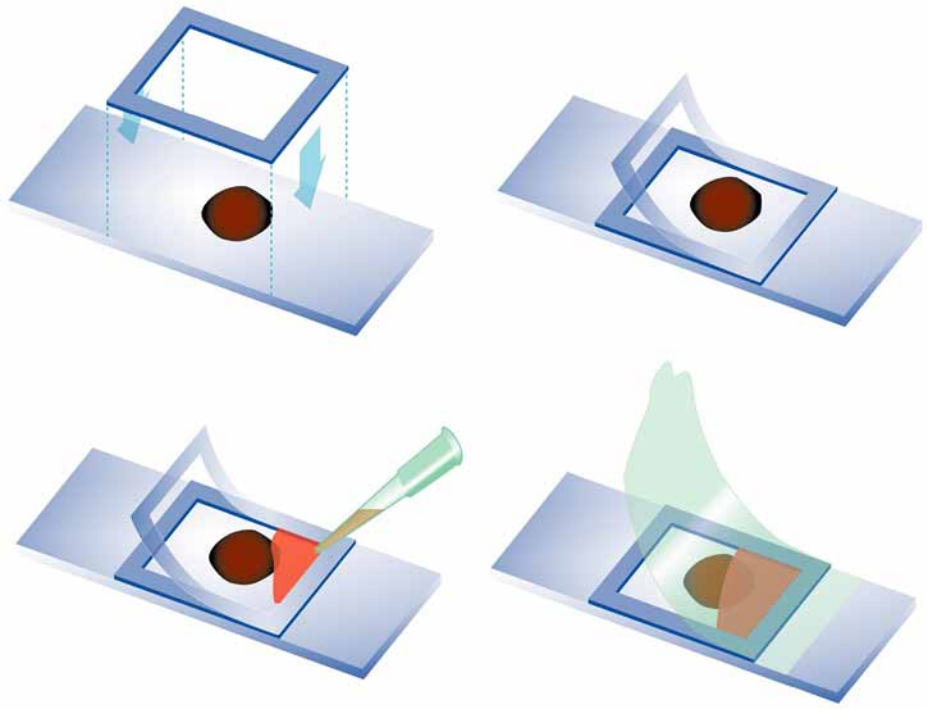

Fig. 1. Overview of frame-seal incubation chambers.

3.3.3. Proteinase K Treatment

Very Important! Two alternative methods for proteinase K digestion are described.

3.3.3.1. Proteinase K Digestion Method A

1. Treat samples with 6 µg/mL proteinase K in PBS for 5 to 60 min at room temperature or at 55°C (no doubt this represents quite a range).
2. After 5 min, look at the cells under the microscope at ×400.
   If the majority of the cells-of-interest exhibit uniform-appearing, small, round "bubbles" or "blebs" or "peppery dots" on the cytoplasmic membrane, then stop the treatment immediately with **step 3**. Otherwise, continue treatment for another 5 min and re-examine.
3. After proper digestion, heat slides on a block at 95°C for 2 min to inactive the proteinase K.
4. Rinse slides in 1X PBS for 10 s.
5. Rinse slides in distilled water for 10 s.
6. Air-dry.

3.3.3.2. Proteinase K Digestion Method B

1. Prepare four or five extra slides of the specific tissue in question. It is especially helpful if the slides are successive sections or very similarly prepared, for the morphology of the various slides must be closely compared later.
2. Prepare an equal number of serial dilutions of proteinase K solution, for example, over the range of 1–6 mg/mL.
3. Decide upon a standard time and temperature for digestion, for example, 15 min at 37°C.
4. Treat the slides in the serial solutions for this standard period.
5. Stop the digestion by heating slides on a block for 2 min at 95°C.
6. Counterstain the slides with hematoxylin or other appropriate stain.
7. Observe slides under a high-power light microscope and look for any morphological change in the cells.
8. Choose the highest concentration of proteinase K that did not result in significant change as the optimized concentration.
9. If all the slides showed change, repeat process with lower concentrations of proteinase K or shorter incubations.
10. If none of the slides showed change, increase concentrations or incubation times and repeat procedure until the proper conditions are found.
11. Once the optimized digestion is determined, use these conditions to process all slides of that particular tissue, fixation method, and thickness of section. Any change in the latter three parameters necessitates re-optimization of the digestion procedure (*see* **Note 7**).

3.4. In Situ *RT-PCR*

3.4.1. DNase Treatment

1. Prepare a RNase-free, DNase solution and add 10 µL of solution to each well.
2. Incubate the slides overnight at 37°C in a humidified chamber. When using liver tissue, this incubation should be extended an additional 18 to 24 h.
3. After incubation, rinse the slides with a similar buffer solution that was prepared without the DNase enzyme.
4. Wash the slides twice with DEPC-treated water.

3.4.2. RT Reaction (see ***Note 8***)

It is advisable to use a premade RT reaction solution that contains all the components necessary to carry out the reaction; one only has to add the mRNA template and a primer (*see* **Note 9**). However, if one desires to prepare his or her own reaction solution, then use the RT reaction solution described in **Subheading 2.**, **step 27**.

1. Add 10 µL of cocktail to each well. Carefully place the cover slip on top of the slide (*see* **Note 10**).
2. Incubate at 42°C or 37°C for 1 h in a humidified atmosphere.

3. Incubate slides at 92°C for 2 min.
4. Remove cover slip and wash twice with distilled water.
5. Proceed with the amplification procedure, which is the same for both DNA- and RNA-based protocols (*see* **Notes 11–13**).

3.4.3. Amplification Protocols (Protocol for Use With Conventional Sealing Technologies (Such as Frame-Seal Incubation Chambers)

1. Prepare an amplification cocktail containing the following: 1.25 µ*M* of each primer (*see* **Note 14**), 200 µ*M* dATP, dCTP, dGTP, dTTP, 10 m*M* Tris-HCl (pH 9.0 at room temperature), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.001% stabilizer (including BSA and gelatin); and 2.5 U of *DNA* polymerase (*see* **Note 15**).
2. Layer 8 µL of amplification solution onto each well with a P20 micropipet so that the whole surface of the well is covered with the solution.
3. If using a single-well slide for a tissue section, add 12–20 µL of the solution to the well.
4. In all cases, be careful—do not touch the surface of the slide with the tip of the pipet.
5. Seal the slides as described herein (**Fig. 1**):
   Place the plastic cover slip on top of the slide, or if using tissue sections, use a second slide instead of a cover slip.
   Carefully seal the edge of the cover slip to the slide.
6. Place on a heat block for 90 s at 92°C to further cure the adhesive.
7. Place slides in an appropriate thermal cycler.
8. Run 30 cycles of the following amplification protocol: 94°C, 2 to 3 min; 45 to 55°C, 1 to 2 min; 72°C, 1 to 5 min depending on the size of amplicons (*see* **Notes 16–18**).
9. After the thermal cycling is complete, open the slides.
10. Carefully, pry off the plastic cover slip. For other sealing technologies, follow the manufacturer's instructions.
11. Place the opened slides on a heat-block at 92°C for 1 min. This treatment helps immobilize the intracellular signals.
12. Wash slides with 2X SSC at room temperature for 5 min, two times.
13. The amplification protocol is now complete and one can proceed to the *in situ* hybridization procedures (*see* **Note 19**).

3.4.4. One-Step RT Amplification

1. If one uses RT enzymes to manufacture complementary (c)DNA, which is subsequently amplified by any of the suitable thermostable RT/DNA polymerases (that both RT and DNA capacity) like *rTth*, use the typical cocktail described in **Subheading 2**, **step 28** for this single-step reaction.
2. This reaction requires a slightly variant thermal cycling profile. Our laboratory uses the following amplification protocol: 42°C, 45 min; 92°C, 3 min; 42°C, 15 min; 92°C, 3 min; 42°C, 15 min; and then, 29 cycles of the following profile: 93°C, 1 min; 53°C, 1 min; 72°C, 1 min.

3.5. Special Applications of In Situ Amplification

3.5.1. In Situ Amplification and Immunohistochemistry

1. Fix cells or frozen sections of tissue, which are already placed on slides, with 100% methanol for 10 min.
2. Wash slides in PBS.
3. After that, labeling of surface antigen(s) can be carried out by standard immunohistochemical method: FITC-conjugated antibody is incubated for 1 h at 37°C, washed, and then cells or tissue section are fixed in 4% paraformaldehyde for 2 h.
4. In various pathology laboratories, many specific surface antigens have been tabulated that can withstand 10% formalin and other routine histopathology procedures and will still bind to specific monoclonal antibodies. If one is using any of these immunohistochemistry panels, then one can also use routinely prepared paraffin sections for the detection of cellular antigens.
5. Then, the tissue is prepared for *in situ* amplification, as described previously.

3.5.2. Multiple Signals, Multiple Labels in Individual Cells

1. As described in the previous section, one can label proteins by FITC-labeled antibodies.
2. Then, one can perform both RNA and DNA *in situ* amplification in the cells.
3. If one is using primers for spliced mRNA and if these primers are not going to bind any sequences in DNA, then both DNA and RT amplification can be carried out simultaneously.
4. Subsequently, products can be identified by using different color probes, with different excitation and emission ranges, resulting in different colors of signal. Currently, there are more than 30 different kinds of color probes available, and one can choose them according to the filter range one has in the laboratory.

3.6. Hybridization

Prepare a solution containing 20–50 pg/µL of the appropriate probe, 50% deionized formamide, 2X SSC buffer, 10X Denhardt's solution, 0.1% sonicated ssDNA, and 0.1% SDS. The following is a convenient recipe for a total volume of 100 µL (*see* **Note 20**):

a. Probe	2 µL
b. Deionized formamide	50 µL
c. 20X SSC	10 µL
d. 50X Denhardt's solution	20 µL
e. 10 mg/mL ssDNA	10 µL
f. 10% SDS	1 µL
g. H_2O	7 µL

1. Add 10 µL of hybridization mixture to each well and add cover slips.
2. Heat slides on a block at 95°C for 5 min.
3. Incubate slides at 48°C for 2 to 4 h in a humidified atmosphere.

3.7. Posthybridization for Peroxidase-Based Color Development

1. Wash slides in 1X PBS twice for 5 min each time.
2. Add 10 µL of streptavidin-peroxidase complex (100 µg/mL in PBS, pH 7.2). Gently apply the cover slips.
3. Incubate slides at 37°C for 1 h.
4. Remove cover slip, wash slides with 1X PBS twice for 5 min each time.
5. Add to each well 100 µL of 3'-amino-9-ethylene carbazole in the presence of 0.03% hydrogen peroxide in 50 m*M* acetate buffer, pH 5.0.
6. Incubate slides at 37°C for 10 min to develop the color—this step should be conducted in the dark. After this period, observe slides under a microscope. If color is not strong, develop for another 10 min.
7. Rinse slides with tap water and allow to dry.
8. Add one drop of 50% glycerol in PBS and apply the cover slips.
9. Analyze with optical microscope—positive cells will be stained a brownish red.

3.8. Posthybridization for Alkaline Phosphatase-Based Color Development

1. After hybridization, remove cover slip, wash the slides with two soakings in 2X SSC at room temperature for 15 min.
2. Cover each well with 100 µL of blocking solution, place the slides flat in a humidified chamber at room temperature for 15 min.
3. Prepare a working conjugate solution by mixing 10 µL of streptavidin–alkaline phosphatase conjugate (40 µg/mL stock) with 90 µL of conjugate dilution buffer for each well.
4. Remove the blocking solution from each slide by touching a paper towel to the edge of the slide.
5. Cover each well with 100 µL of freshly prepared working conjugate solution and incubate in the humid chamber at room temperature for 15 min. Do not allow the tissue to dry out after adding the conjugate.
6. Wash slides by soaking in buffer A for 15 min at room temperature twice.
7. Wash slides once in alkaline substrate buffer at room temperature for 5 min.
8. Prewarm 50 mL of alkaline-substrate buffer to 37°C in a Coplin jar. Just before adding the slides, add 200 µL of NBT and 166 µL of BCIP. Mix well.
9. Incubate slides in the NBT/BCIP solution at 37°C until the desired level of signal is achieved (usually from 10 min to 2 h). Check the color development periodically by removing a slide from the NBT/BCIP solution. Be careful not to allow the tissue to dry out.
10. Stop the color development by rinsing the slides in several changes of deionized water. The tissue may now be counterstained.

3.9. Posthybridization for Digoxigenin-Labeled Probe

1. Use antidigoxigenin–peroxidase solution (1:250 dilution in PBS).
2. Incubate for 2 h at 37°C.

3. Wash three times with PBS.
4. Develop color with AEC, as described in **Subheading 2.**, **step 17**.

3.10. Posthybridization for Fluoresent Color Probes

1. Wash the slides with PBS three times.
2. Mount the slides with 50% glycerol/PBS with antifade reagent.
3. Observe under UV microscope with appropriate filter range.

3.11. Counterstaining and Mounting

An appropriate counterstain can be used for the cells that are not carrying the amplified signals. This step is critical, and one has to work out the fine detail of the counterstains before actually applying on the slides that were used for the aforementioned protocols.

1. Rinse in several exchanges of a buffer.
2. Dehydrate the sections through graded ethanol series (50%, 70%, 90%, and 100% (v/v) for 1 min each).
3. For permanent mounting, a water-based mounting medium can be used (*see* **Subheading 2**, **step 38**).
4. Apply one drop of mounting medium per each 22 mm to cover slip area.
5. The slides may be viewed immediately, if you are careful not to disrupt the cover slip. The mounting medium will dry after sitting overnight at room temperature.

3.12. Validation and Controls

1. Run two or three sets of experiments in multiwelled slides simultaneously, for not only must one validate amplification, but one must also confirm the subsequent hybridization/detection steps as well.
2. In all amplification procedures, use one slide as a control for nonspecific binding of the probe. Here we hybridize the amplified cells with an unrelated probe.
3. We also use HLA-DQα, or β-actin probes and primers with human peripheral blood mononuclear cells as positive controls to check various parameters of our system.
4. If you are using tissue sections, a cell suspension lacking the gene of interest can be used as a control. Then retrieved following the amplification procedure.
5. The cell suspension can then be analyzed with the specific probe to see whether the signal from the tissue leaked out and entered the cells floating above.
6. In the case of RT *in situ* amplification, one can use β-actin, HLA-DQα, and other endogenous-abundant RNAs as the positive markers.

4. Notes

1. If one chooses to use special slides that have Teflon coatings that form individual "wells," then they can be purchased from one of the following sources. These slides are not necessary to purchase, and we no longer use them and prefer to use

Frame-Seal Incubation Chambers, instead. However, the source of the material for both kinds of chamber system is MJ Research, Inc., Waltham, MA (info@mjr.com).

2. Sterile technique must be practiced at all times during the culturing and prefixation parts of this procedure. Sterility is especially important in handling cell cultures, both to protect the investigator and to avoid introducing microbial contamination of the cell culture system. Such contamination is often the cause of test failure. The human peripheral blood used in this procedure may be infectious or hazardous to the investigator. Proper handling and decontamination and disposal of waste material must be emphasized.
3. If one uses two slides with two Teflon-coated sides facing one another rather than a slide and a cover slip, this allows for a double-thick reaction chamber that can hold a bit more amplification cocktail.
4. It is possible to use frozen sections for *in situ* amplification; however, the morphology of the tissue after the amplification process generally is not as good as with paraffin sections. Apparently, the cryogenic freezing of the tissue, combined with the lack of paraffin substrate during slicing, compromises the integrity of the tissue. Usually thicker slices must be made, and the tissue can "chatter" in the microtome. As any clinical pathologist will relate, definitive diagnoses are made from paraffin sections, and this rule-of-thumb seems to extend to the amplification procedure as well. The exception to the rule is when one wishes to use immunohistochemical techniques to detect additional signals in the cells. Some of these techniques require frozen sections and, in such a circumstance, the use of frozen sections is appropriate. However, there are new fixatives—such as "Permiofix" from Ortho Diagnostics—that preserve cell surface antigens in fixed tissue. These matters are explained further in a later section on immunohistochemistry.
5. Difficulties will often be experienced in slicing sections thinly because the tissue is either too cold or it is insufficiently frozen. This is remedied by use of pathologist's freezing spray—merely blast the central area with a few quick bursts of spray, wait a few moments and proceed. If instead the tissue will not slice at all, it could be that the tissue is too solidly frozen. To remedy this problem, allow the tissue to equilibrate overnight at –70°C while it is mounted on the disk in the cryostat.
6. Some RNA signals may not be very stable at high temperatures. Therefore, we use the shorter incubation times (5–10 s) with RNA targets and longer times (90–120 s) with DNA signals. One may need to experiment with different periods to find the best heat treatment for the specific tissue and target.
7. The time and temperature of incubation should be optimized carefully for each cell line or tissue-section type. With too little digestion, the cytoplasmic and nuclear membranes will not be sufficiently permeable to primers and enzyme, and amplification will be inconsistent (or nonexistent). With too much digestion, the membranes will lose integrity and leak amplicons, making surrounding cells falsely positive, or high background or poor morphology will result. Often with

excessive digestion, many cells will show peri-cytoplasmic staining, which represents leaked signal contaminating cells in which no positive signal actually exists. Attention to detail with the proteinase K digestion often can mean the difference between success and failure in an experiment, and this digestion should be practiced on extra sections by anyone attempting to conduct this protocol for the first time. In our laboratory, proper digestion parameters vary considerably with tissue type. Typically, lymphocytes will require 5 to 10 min at 25°C or room temperature, central nervous system tissue will require 12 to 18 min at room temperature, and paraffin-fixed tissue will require between 15 and 30 min at room temperature (these times can be accelerated by using higher temperatures of incubation, up to 55°C). However, the periods can vary widely, and one has to optimize the conditions by carrying out careful reactions with control cells.

8. Avian myoblastosis virus reverse transcriptase (AMVRT) and Moloney murine leukemia virus reverse transcriptase (MuLVRT) give comparable results in our laboratory. Other RT enzymes also will probably work. However, it is important to read the manufacturer's descriptions of the RT enzyme and to make certain that the proper buffer solution is used (which may be different from that recommended here). One can use an oligo(dT) primer, random primer such as pd(N)6, or a specific primer that only anneals to the mRNA of interest.
9. The preservation of intact mRNA is of primary importance and the success of first-strand cDNA synthesis depends upon the integrity of the mRNA of interest.
10. All reagents for RT *in situ* PCR should be prepared with RNase-free water (i.e., DEPC-treated water). In addition, the silanized glass slides and all glassware should be RNase-free, which we insure by baking the glassware overnight in an oven at 250 to 300°C before use in the RT procedure.
11. Choices of primers include approx 2 μg of $pd(N)_6$, 1 ng of $pd(T)_{12–18}$, or 15 p*M* of mRNA-specific primers.
12. The RT-PCR cocktail must be preserved from degradation and should be kept on ice to minimize the formation of nonspecific first-strand products.
13. In all RT reactions, it is desirable to reverse-transcribe only relatively small fragments of mRNA (<1500 base pairs). Larger fragments may not completely reverse-transcribe because of the presence of secondary structures. Furthermore, the RT enzymes—AMVRT and MuLVRT, at least—are not very efficient in transcribing large mRNA fragments. For DNA amplification, we routinely amplify genes of 300 to 500 base pairs, and we find this size works well. Recently, many articles have been published with new combinations of polymerase enzyme and buffers that allow efficient amplification of much larger fragments of DNA or cDNA (as much as 50 kb or more). We see no reason to prevent these new techniques from being adapted to *in situ* amplification.
14. Regarding the design of primers, the following are several additional points one should keep in mind: use of a computer-aided design program such as: http://www.bioinformatics.vg/biolinks/bioinformatics/PCR%2520and%2520Primer%2520Design.shtml combined with the data resources offered by GENBANK (http://www.ncbi.nlm.nih.gov/entrez/), can often lead to superior

primers. The length for both sense and antisense primers should be 18 to 22 base pairs. At the 3'-ends, primers should contain GC-type base pairs (e.g., GG, CC, GC, or CG) to facilitate complementary strand formation (a GC-type bond will

in other words, the primer can grab on and anneal more readily). The preferred overall GC content of the primers is from 45 to 55%. Try to design primers so they not form intrastrand or interstrand base pairs. Furthermore, the 3'-ends should not be complementary to each other, or they will anneal to one another and form primer-dimers. One can design an RT-primer so that it does not contain secondary structures, and it is not complementary to RNA that will form secondary structures.

15. Other thermostable polymerase enzymes also have been used quite successfully.
16. Some RT enzyme work best at 72°C and or other temperatures, and one should check this before setting the cycles in the thermocycler.
17. Annealing temperatures for reverse transcription and for DNA amplification can be chosen according to the following formula:

    Tm of the primers = 81.5°C + 16.6 (Log M) + 0.41 (G + C%) – 500/n

    Where n = length of primers, *M* = molarity of the salt in the buffer, usually 0.047 *M* for DNA reactions and 0.070 *M* for RT reactions.
    If using AMVRT, the value will be lower according to the following formula:

    Tm of the primers = 62.3°C + 0.41 (G + C%) – 500/n

    Usually, primer annealing is optimal at 2°C greater than its Tm. However, this formula provides only an approximate temperature for annealing because base-stacking, near-neighbor effect, and buffering capacity may play a significant role for any particular primer. Optimization of the annealing temperature should be conducted first with solution-based PCRs. It is particularly important to know the optimal temperature before attempting to conduct *in situ* amplification, for several reasons. First, the highest-fidelity annealing occurs at relatively high temperatures for any primer, and maximum specificity goes hand-in-hand with high fidelity. If the annealing temperature is two low, spurious priming can occur, where the match between primer and template is not exactly homologous. Nonetheless, annealing occurs anyway, because DNA has an enormous affinity for being double stranded at lower temperature—even when there are base-pair mismatches. Then, these false primes get extended by polymerase enzymes, and spurious amplification products result. However, if the annealing temperature is too high, there is very little or no annealing of the primers to the template. Then, there is no place for the polymerase enzyme to grab on for extention, and very little or no amplification of any kind results. Second, annealing temperatures are important because *in situ* reactions, in general, are neither as robust nor as efficient as solution-based ones. We hypothesize this is because primers cannot easily access DNA templates inside cells and tissues because numerous membranes, folds, the tissue matrix, and other small structures can prevent primers from binding homologous sites as readily as they do in solution-based reactions. Thus, the temperature of annealing

should be just right to make the best of a difficult situation. Last, but not least, many researchers will go to great efforts to develop protocols that include a "HOT start," which can help prevent false priming on the on the first cycle ***(3–6)***. Similar techniques have evolved using dimethyl sulfoxide, formamide, and anti-*Taq* antibodies (i.e., Clontech's TaqStart). However, it has been our experience that better results can be obtained by instead concentrating on the optimization of annealing temperature because this optimization can minimize false priming throughout the thermal cycling regime, not just on the first cycle. Thus, it is worthwhile taking the trouble to run reactions in solution-based reactions with extracted DNA/RNA to optimize the annealing temperatures. What one seeks is an annealing temperature that results in thick bands in electrophoresis gels without an abundance of nonspecific amplicons. However, bear in mind that one is looking for the highest temperature that will give these sorts of results—as little as 1°C can make a substantial difference. Alternatively, if one does not have the time to fully optimize the annealing temperature, one can use a "touchdown" protocol, in which the annealing temperature is initially set rather high, but it ratchets down by approx 0.5°C with each subsequent annealing step for the first 10 to 20 cycles. The idea here is to first create a number of high-fidelity amplicons that get geometrically amplified in the subsequent cycles—in other words, one increases the signal-to-noise ratio for better results, even though the final annealing temperature might be substantially below the optimum.

18. There is much debate as to whether a hot start helps to improve the specificity and sensitivity of amplification reactions. In our laboratory, we find the hot start adds no advantage in this regard; rather, it adds only technical difficulty to the practice of the *in situ* technique. However, if one prefers to use a hot start, we recommend trying a variation suggested by Stuart Isaacson. Dr. Isaacson—whose laboratory specializes in archival brain tissue amplified and probed for RNA virus—suggests the following:

    "It is our experience that a hot start is helpful when the amplification reaction does not work at all, or when the efficiency of recovery is low. By keeping separate the template from the amplification reaction mixture at temperatures below annealing, less stringent 'competitive' binding of primers to undesired sequences is minimized. This can allow a higher yield of amplification product. Thus, a hot start is not necessary routinely, but it is a way to optimize amplification efficiency and consequent signal intensity."

19. Sometimes it is desirable to recover samples of the amplified products from *in situ* reactions. For example, perhaps a sample is needed to sequence amplicons so that various alleles of a gene—or even various expressions of a gene—can be distinguished. Perhaps an investigator wishes to clone the gene in question to obtain larger quantities for future study. But perhaps the most exciting application is in the field of developmental biology, for amplification products can help in determining what proteins are involved in the processes of cellular differentia-

tion and organogenesis. For example, sections of embryos can be taken at various stages of development, and either specific primers or oligo(dt) primers can be used to identify the gene expression that is occurring in particular index cells. If one reserves and stores the supernatant after amplification, then one go back and re-amplify the gene-of-interest once the specific phase of differentiation has been identified in the index cells following hybridization of the whole tissue. In this circumstance, one might even use a micromanipulator to recover the specific cells, in order to study gene expression even more closely. By simply reserving the amplification cocktail after the thermal cycling procedure, recovery of the amplicon usually can be achieved. It has been our experience that there is usually a small amount of leakage out from the cells into the amplification cocktail during amplification, but with proper proteinase K digestions, there is very little or no leakage into other cells in the tissue sample. We have collected abundant data on this matter, in particular by running samples of the supernatant in electrophoretic gels after amplification. We have found that amplified products usually can be detected by Southern blotting but not by ethidium bromide labeling because the signal is very weak. However, this product usually can be reamplified in a subsequent solution-based reaction using the same primers as the *in situ* reaction (or a nested primer pair), and this provides sufficient quantities of the amplicon for subsequent cloning or analysis. We hypothesize that the leakage of signal in the original *in situ* reaction tends to occur late in thermal cycling, as the signal recovered from the supernatant is almost invariably quite weak (at least when the proteinase K digestion was properly optimized). In the latter cycles of the PCR procedure, the geometric nature of the amplification makes the concentration of the amplified product very high at the original locus of the target, and it is reasonable to assume that some small fraction of the amplified signal could drift away from this locus and diffuse out of the cell. This would be particularly true for those positive cells whose nuclear or cytoplasmic membranes were sliced open by the blade of the microtome, such that a primary containment "vessel" of the signal was violated. However, if this diffusion were occurring in significant quantity or early in the amplification process, we would expect to see strong signals in the supernatant—manifested both in the agarose gel and in inter-cytoplasmic staining after hybridization—because subsequent amplification would have occurred in the supernatant. In fact, we do not see strong signals, except in those circumstances in which there was excessive digestion with proteinase K. Last but not least, we have encountered no evidence to indicate that leaked signals enter into other cells to deliver false-positives upon subsequent hybridization, as we believe this would require fairly large membrane pores. Rather, we hypothesize that during the optimized proteinase K digestion, there is only partial digestion of certain transmembrane proteins which results in the formation of semi-permeable pores. These pores selectively allow positively charged molecules to pass, but they tend to block negatively charged molecules. Therefore, the *Taq* enzyme and single-stranded small primers can enter the cell, but double-stranded

amplicons (which are highly negatively charged) cannot readily pass. Therefore, false signals do not enter the cells. To recover the supernatant, one simply can pry open a corner of the plastic sealant and siphon off the solution. This solution can be used for analyses or re-amplification. Ideally, one wishes to recovery as much of the supernatant as possible, and that it would comprise 10 to 15 μL of cocktail.

20. The salmon sperm should be denatured at 94°C for 10 min before it is added to the hybridization buffer. 2% BSA can be added if one is observing nonspecific binding. One can add 10 μL of 20% BSA solution and reduce the amount of water.

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II

Research and Clinical Applications

8

Detection and Sizing of Telomeric and Other Simple Repeats by Dideoxy-PRINS

Jørn Koch

Summary

The protocol is suited for the quantitative and qualitative detection of simple repeat target DNA composed of three or fewer of the four bases A, C, G, and T. A consequence of the lacking base(s) is that such DNA can be synthesized from nucleotide mixtures containing the particular bases as dideoxynucleotides. Most genomic DNA contains all four bases and can therefore not be synthesized from such a nucleotide mixture. The combined effects of probe specificity and selective DNA synthesis from the nucleotide mixture improves the signal-to-noise ratio for such targets approximately an order of magnitude, enabling the detection of exceedingly small hybridization targets (e.g., variant telomeric repeat variants embedded in, or situated next to, the main repeat), provided that they present enough template for the DNA synthesis.

Key Words: PRINS; telomere; telomeric repeat; trinucleotide repeat.

1. Introduction

Organisms are made of tissues, and tissues are made of cells. All tissues are composed from a mixture of many types of cells. On a genetic level, these cells differ in their gene expression and, in cancers, also in their DNA. Because the performance of any tissue and, ultimately, the organism depends on the behavior of its constituent cells, a thorough understanding of biological processes in biology and in pathology requires studies of the individual cells and the biomolecules within the cells. Dominant molecular biology methods do not provide this insight because they rely on the analysis of biomolecules isolated from extracts of pools of cells. To get the full picture, it is necessary to obtain data on the individual cells in a format that also gives spatial information on the biomolecules inside the cells and the cells inside the tissues. In essence, this means that cells and biomolecules should be studied *in situ*, which has been possible since the introduction of *in situ* hybridization and immuno-

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histochemistry. Unfortunately, both techniques have a limited sensitivity (cannot visualize ultimately low amounts of targets) and resolution (cannot see ultimately small features of the targets). I therefore undertook the development of a new technology with the prospect of providing single molecule DNA and RNA detection *in situ* at single-nucleotide resolution approximately two decades ago ***(1–5)***. The underlying principles of the new technology were that short oligonucleotides were used as probes for the target definition and that DNA synthesis at sites binding the probes was used for target visualization. With oligonucleotide probes being sensitive down to single-base variations in the target sequence, it should be possible to identify point mutations (or single-nucleotide polymorphisms) in target sequences, and with the DNA synthesis being capable of proceeding for tens of kilobases in vitro, it should be possible to generate enough DNA from a single priming event for it to become visible by the microscope. This potential has now been turned into a series of protocols, as evidenced by this book. In most protocols, a number of priming events per target are required to make it visible above background because of the low and variable frequency of priming events from endogenous 3'-ends of DNA, either generated during the life of the cell or as a consequence of the cell preparation. Thus, whereas repeated targets enable multiple priming events from a single probe, standard detection of single-copy genes has required the use of a cocktail of probes for that gene ***(6,7)***. However, there are two ways in which to obtain probe-specific *in situ* DNA synthesis in the absence of endogenous DNA synthesis, one of which is the dideoxy-PRINS described here. The other is rolling-circle primed *in situ* labeling (PRINS; *see* Chapter 5).

2. Materials

2.1. Basic Ingredients and Instrumentation

1. Chromosome spreads (preferably freshly made; *see* **Note 1**).
2. Cover slips.
3. Coplin jars or other suitable containers for washing the slides.
4. Standard nucleoside triphosphates, dideoxy nucleotides (Lithium salt, e.g., Roche).
5. Hapten- or fluorochrome-labeled nucleotides (digoxigenin-2'-deoxyuridine 5'-triphosphate [dUTP], biotin-dUTP, fluorescein-dUTP, rhodamine-dUTP, etc.; e.g., Roche).
6. Glycerol (*see* **Note 2**).
7. Thermostable DNA polymerase that will accept labelled nucleotides (e.g., Tth or *Taq* DNA polymerase) and 10X polymerase buffer (enzyme and buffer are supplied together by Roche and most other suppliers). For convenience, the 10X buffer may be mixed with an equal volume of glycerol into a 5X buffer that will not freeze at –20°C.

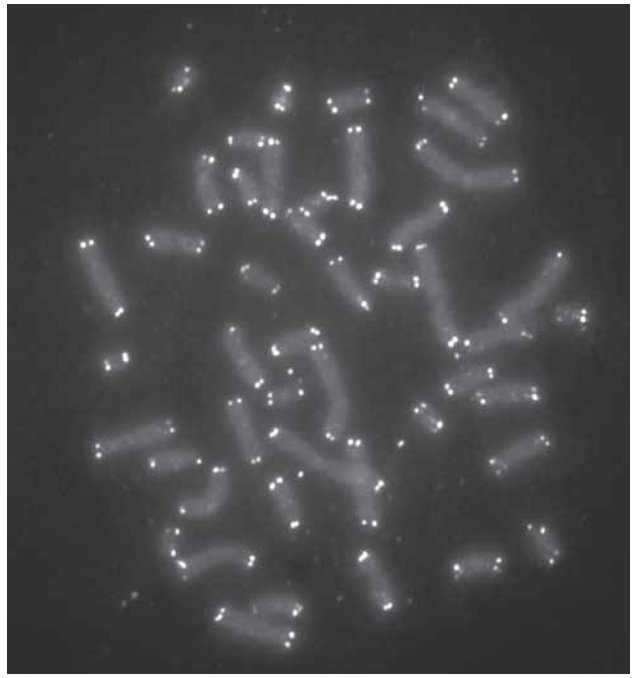

Fig. 1. ddPRINS on a spread of human chromosomes. Each chromosome is made of two chromatids, and each chromatid has two ends, so the number of targets/signals/cell is in principle 184. However, signals on neighboring chromatids may appear fused. The outline of the chromosomes is also visible, mainly owing to autofluorescence from the chromatin.

8. 5 *M* NaCl.
9. 500 m*M* Ethylene diamine tetraacetic acid (EDTA).
10. 20X Standard saline citrate (SSC).
11. 20% Tween-20.
12. Ethanol.
13. Nonfat dry milk (powder).
14. Fluorochrome labeled anti-digoxigenin for detection of digoxigenin labeled PRINS product (Fab fragment, Roche).
15. Fluorochrome-labeled streptavidin for the detection of biotin-labeled PRINS product (Roche, Vector Laboratories).
16. Antifade solution: Vectashield (Vector Laboratories) or *p*-phenylenediamine-dihydrochloride (Sigma).
17. Counterstain: either propidium iodide (Sigma) or DAPI (Sigma).
18. If denaturation and hybridization are performed consecutively, either a special PRINS/*in situ* PCR machine (from Hybaid, MJ-Research or Techne) or two incu-

bators (thermoblocks, waterbaths) are needed. They must provide a metal surface to ensure good heat transfer so that denaturation at 94°C and hybridization at 55–65°C can be obtained. To ensure the correct temperature, it may be helpful to cover the slide and the hot plate with an insulating lid. If slides are already denatured, one incubator is sufficient.

19. Fluorescence microscope with standard excitation and emission filters (e.g., 81000 filter set from Chroma Technology).

2.2. Mixtures

1. Ice cold (–20°C) ethanol series (70%, 90%, and 99% v/v) for PRINS on predenatured slides.
2. 10X dNTP for dideoxy-PRINS (with ddGTP for telomere staining with the Telo2 primer ($CCCTAA_7$ ***[1,2,8,9]***): 1.0 m*M* each of dATP, dCTP; 1.0 m*M* ddGTP; 0.1 m*M* of labeled dUTP; mix in 50% glycerol. Can be stored at –20°C for 1 yr (*see* **Note 3**).
3. Reaction mixture (60 µL total): 6 µL 10X buffer (or 12 µL 5X), 6 µL of 10X dNTP, 1 µL (approx 1 µg) oligonucleotide, 1 µL (approx 1U enzyme), and 38 µL (or 33 µL) ultrapure water. The polymerase should not be added until the mixture is actually used, but the mixture without polymerase may be prepared weeks in advance and stored at –20°C.
4. Stop buffer: 50 m*M* NaCl, 50 m*M* ethylene diamine tetraacetic acid, pH 8.0. This buffer can be stored at room temperature for months.
5. Wash buffer: 4X SSC, pH 7.0 (1X SSC: 150 m*M* NaCl, 15 m*M* sodium citrate), 0.05% Tween-20. This buffer can be stored at room temperature for months.
6. Blocking solution: 5% (w/v) nonfat dry milk dissolved in washing buffer. Centrifuge for 2 min in an Eppendorf centrifuge and use supernatant. This solution can be stored at –20°C for years (*see* **Note 4**).

3. Methods

3.1. Pretreatment of Samples

1. The main limitation to the intensity of the signal is the extent of the chain elongation *in situ*. Often this stops after 150 to 200 bases, presumably as a result of increased tension in the template DNA as the DNA synthesis progresses. The increase in tension may be prevented or reduced if the preparation also is treated with an enzyme that breaks the DNA at defined positions without ruining the template, for example, a rare cutting restriction enzyme like *Sal*I, may increase signal intensity up to 10-fold. For this pretreatment, the slide should be incubated with 1 U restriction enzyme for 1 h at the temperature optimum of the enzyme. It may be necessary to seal the cover slip to the slide because glycerol cannot be included to prevent evaporation (glycerol enhances DNA polymerases and exonucleases without apparent side-effects but induces star activity of restriction enzymes). After the pretreatment, the slide should be dehydrated in an ethanol series (70%, 90%, and 99% for 3 min each), after which it is removed from the

99% ethanol, drained, and air-dried. This pretreatment has no visible effect on the telomere staining, presumably because the template DNA is at the end of the chromosome anyway but may be useful for the detection of internal repeats.

2. The target DNA can be denatured as part of the PRINS proper according to the protocol in **Subheading 3.2.** Alternatively, the slide may be denatured before the reaction (e.g., in 50–70% formamide, at 70°C, for 2 min). It is important that the slide is immediately quenched in a –20°C ethanol series (70%, 90%, and 99% for 3 min each) to fix the target DNA in the denatured configuration. After the dehydration, the slide is removed from the 99% ethanol, drained, and air-dried. **Step 2** in the protocol is then bypassed for the PRINS proper.

3.2. PRINS Proper

1. Decide how large a region of the slide should be analyzed and choose a cover slip and an amount of reaction mixture that fits the area. Use 1-µL reaction mixture for each mm of cover slip length. To cover a standard slide completely, prepare a 60-µL reaction mixture and cover with a 24- × 60-mm cover slip. To cover a smaller area, prepare less reaction mixture (and use a smaller cover slip).
2. As soon as the reaction mixture has been spread with the cover slip, denature the slide by placing it on a hotplate covered with a lid for 4 min at 94°C (with the automated incubators the temperature could be a bit lower because they often operate from a simulated slide function, and the temperature inside the slide is slightly lower than the surface temperature of the incubator).
3. Either lower the temperature to between 55 and 65°C or transfer the slide to this temperature and incubate for 5 to 60 min for probe annealing and chain elongation (*see* **Note 5**).
4. Place the slide in preheated stop buffer at the temperature used in **step 3** for 1 min to terminate the PRINS reaction.
5. Transfer the slide to 50 mL of wash buffer and wash for approx 3 min at room temperature.

The reaction can be paused at this step and the slide stored overnight in wash buffer at 4°C.

6. If fluorochrome-labeled nucleotides have been used; the slide can now be counterstained, mounted, and evaluated under the microscope. If digoxigenin- or biotin-labeled nucleotides have been used, they need to be visualized with antidigoxigenin antibody or streptavidin.

3.3. Visualization of Digoxigenin- or Biotin-Labeled PRINS Products

1. Apply 50 µL of fluorochrome-conjugated antidigoxigenin (or fluorochrome-conjugated streptavidin) in blocking solution (2 ng/µL) to the slide. Incubate under a cover slip for 30 to 60 min in the dark.
2. Wash twice for 5 min in 50 mL of washing buffer. The slide is now ready for counterstaining and mounting.

3.4. Counterstaining and Mounting

1. Blue counterstaining of DNA is obtained by including DAPI in the antifade solution. Mount the slide in 20 µL of antifade solution containing 0.4 µ*M* DAPI.
2. Red counterstaining of DNA is obtained by including propidium iodide in the antifade solution. Mount the slide in 20 µL of antifade solution containing 0.5 µg/mL propidium iodide.

3.5. Quantitative PRINS

With the PRINS design, high concentrations of small probes that penetrate the specimen well can be used, making targets effectively saturated and the reaction quantitative. This is particularly true for the dideoxy-PRINS, which is used for measuring the size of telomeric repeat domains at individual chromosome ends, as well as for sizing of other simple repeat domains that vary in size, such as trinucleotide repeats.

Evaluation of quantitative PRINS reactions can be done in either of two ways. One way is by counting signals and calculating the fraction of potential targets actually stained (the author typically counts 100 potential targets): The higher the staining frequency, the larger the target ***(8)***. With dideoxy-PRINS, this often becomes impractical because the staining efficiency in many cases is close to 100% (e.g., 99.8% for telomeric repeats in normal blood lymphocytes). In such cases, it is preferable to measure the light intensities of the PRINS signals instead and average those over 10 metaphases. I use the pre-existing image analysis program QUIPS from VYSIS. This program was designed for CGH and compares the intensity of a test sample to the intensity of a reference sample. For PRINS analysis, the signals are designated as "tester" and the DAPI counterstain as "reference," and the result is thus a signal-to-counterstain ratio that is directly proportional to the amount of target sequence ***(1,2)***. The adaptation to the program has the derived advantage that some of the factors causing artificial variation in signal intensities (e.g., uneven illumination) have a similar influence on the counterstain, leaving the ratio less affected than the absolute values (*see* **Note 6**).

4. Notes

1. The PRINS technique relies strongly on the intact nature of the target sequences—nicked or broken DNA is a poor template for chain elongation *in situ*—and freshly prepared slides are the preferred starting point for an optimal reaction. If good slides cannot be prepared, accessibility can be increased by treatment with a proteinase (DNase-free), vulnerable chromatin can be stabilized by fixation with paraformaldehyde, and damaged DNA can be repaired with a DNA ligase ***(10)***. The chromatin in "aged" slides may be so hardened that it requires long incubations to denature it by heat, for example, 10 min for a slide aged 1 mo at room temperature. An occasional problem in PRINS is "self-labeling" of certain

regions of satellite DNA. In human chromosomes, satellite III on chromosome 9 and, more rarely, satellite II on chromosomes 1 and 16 may self-label. This self-labeling is somewhat dependent on the primer but may occur with any primer. Some primers always induce the self-labeling, but with most primers it is only seen in low quality chromosome spreads.

2. Glycerol prevents evaporation and enhances the reaction in concentrations of 5 to 20%. If the nucleotides, and possibly the buffer, are stored in 50% glycerol, that concentration may be reached without the addition of extra glycerol (the oligonucleotide probes also may be stored in 50% glycerol, in which case all reagents in the reaction mixture (except for the water) can be pipetted directly from –20°C).
3. Reaction mixture without polymerase may be stored at –20°C for months.
4. When in use, it is preferable to store the blocking solution at 4°C and not use it for more than 1 wk (the milk turns sour with time, which may also happen to the milk powder if it sucks water from the air).
5. Probes for simple repeats anneal rapidly, more complex probes more slowly, and chain elongation is virtually instantaneous; therefore, the annealing time determines the incubation time.
6. In collaboration with Peter Lansdorp, DAKO has released an analysis program that is tailor-made for telomere signal quantification. This program operates with absolute intensity values.

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9

PRINS Evaluation of Chromosome Instability in Mammalian Cells by Detection of Repetitive DNA Sequences in Micronuclei

Antonella Russo

Summary

Micronuclei are indirect but reliable indicators of chromosome damage, formed at the mitotic division after either chromosome loss or chromosome breakage events, and identified at interphase as small bodies of extra-chromatin in the cytoplasm of mammalian cells. In this chapter, the procedures for single and dual-color primed *in situ* labeling detection of repetitive DNA sequences in micronuclei are described; the approach allows making inferences on the mechanism of micronucleus formation by considering the centromeric, pericentromeric and telomeric sequences as indicators of chromosome integrity into the micronuclei.

Key Words: Micronucleus; PRINS; chromosome aberrations; aneuploidy; chromosome fragments; mutagenesis; cancerogenesis.

1. Introduction

1.1. The Micronucleus Assay

In mammalian cells, small bodies of extra-chromatin denoted micronuclei can be observed in the cytoplasm at interphase and represent a rare abnormality consequent to genetic damage. Micronuclei are formed at the mitotic division by two different pathways (**Fig. 1**): (1) a pre-existing chromosome fragment lacking the centromere and, therefore, unable to move toward the spindle poles, can decondense into a micronucleus if excluded from the daughter nuclei (**Fig. 1A**); or (2) micronuclei can be derived from a chromosome lagging at anaphase, as described in **Fig. 1B**. Micronuclei are indirect but reliable indicators of chromosome damage: their frequency increases in either in vivo or in vitro cell populations exposed to different radiations or chemicals.

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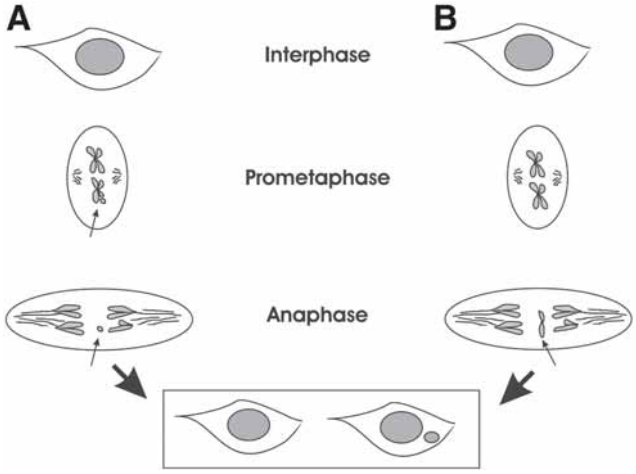

Fig. 1. The two pathways leading to micronucleus formation after a mitotic cell division. (**A**) top to bottom: a cell carrying a chromosome break (indicated by the black arrow) cannot control the segregation of the acentric fragment, which can be excluded from both daughter nuclei. In the cell progeny, depicted in the frame at bottom, one cell will carry a micronucleus. (**B**) top to bottom: a chromosome segregation error can occur during the mitotic cell division; if a chromatid is not able to keep or maintain contact with the spindle, it will lag at anaphase and possibly will be excluded from both daughter nuclei. In the cell progeny, depicted in the bottom frame, one cell will carry a micronucleus. Noteworthy, the two mechanisms lead to the same visible result, unless the micronucleus organization is not defined by molecular cytogenetics; for example, the micronucleus will contain the centromeric sequence only after pathway (**B**). For details *see* **Fig. 2.**

Thirty years ago, a micronucleus assay in in vivo proliferating rodent cells was proposed as a simple experimental procedure for the identification of chemical and physical agents affecting genome stability ***(1,2)***. In contrast to the direct chromosome aberration analysis in metaphase spreads, the micronucleus assay is fast and does not require skilled personnel. For these reasons, a great effort has been put to validate and improve the methodology with respect to the original proposal. At present, the micronucleus assay is a well-established and widely applied approach to evaluate the effects of potential mutagenic agents in several cell types in vivo ***(3)*** and in vitro ***(4)***.

1.2. Application of Molecular Cytogenetics to the Micronucleus Assay

Because micronuclei originating from the two pathways depicted in **Fig. 1** are morphologically identical, the preferential mechanism of action (i.e.,

clastogenic vs aneuploidogenic activity) of many agents of environmental importance cannot be directly defined. With the advent of molecular cytogenetics, different strategies were used to distinguish between micronuclei originating from chromosome breaks or chromosome loss: the key DNA sequences are the centromeric and telomeric sequences because their presence in the micronucleus can demonstrate the preservation of chromosome integrity. Centromere-bearing micronuclei derive from chromosome loss (**Fig. 1B**) and can be considered for simplicity as whole chromosomes, even if the possibility that they harbor a chromosome with terminal deletion cannot be excluded. However, in this remote situation, the reason for micronucleus formation still would be the segregation error, as demonstrated by the presence of the centromeric sequence. Remember that the alternative modality of micronucleus formation, based on a clastogenic action, implies that the chromosome fragment lacks the centromere (it would be an acentric fragment; **Fig. 1A**). For a better definition of micronucleus content and, consequently, of the mechanisms of induced chromosome damage in mammalian somatic and germ cells, dual-color approaches for the simultaneous localization of centromeric, pericentromeric, and telomeric sequences can be applied. **Figure 2** exemplifies the possible molecular patterns expected in micronuclei after single- and dual-color detection of the aforementioned repetitive DNA sequences.

1.3. PRINS Characterization of Micronuclei

The advantages related to the application of primed *in situ* labeling (PRINS) with respect to fluorescence *in situ* hybridization (FISH) are well known, and they are discussed in detail elsewhere in this book (*see* Chapters 1, 3, and 6). In particular, the background noise is lower with PRINS than with FISH, which is crucial in interphase analysis when the discrimination of true reaction signals from aspecific fluorescent spots is difficult. Accordingly, in the micronucleus assay, higher sensitivity and sensibility were found after PRINS than after FISH ***(5)***. A second important advantage of PRINS is that the protocol can be applied easily on cell types of different species, provided that knowledge of the target DNA sequence exists, because the labeling procedure is based on the use of oligonucleotide primers, which are available from many companies or institutional facilities. This aspect is very important because the optimization of commercially available probes for FISH is mainly restricted to diagnostic purposes and, therefore, to the human genome.

In this chapter the procedures for single- and dual-color PRINS detection of repetitive DNA sequences in micronuclei of mammalian cells are described. In addition, a guide to the interpretation of the results and to the choice of most appropriate labeling combinations is provided.

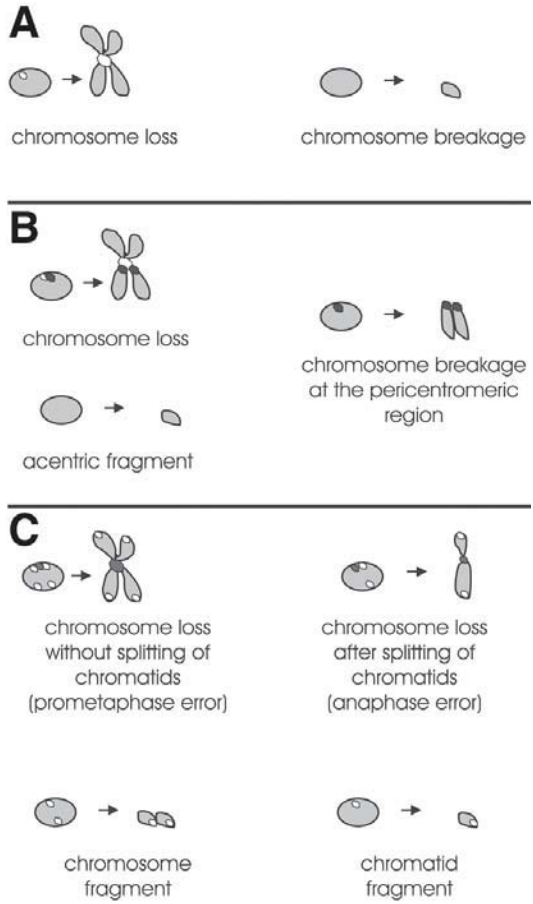

Fig. 2. The detection of repetitive sequences in micronuclei allows one to infer the mechanism of origin: (**A**) the detection of centromeric sequences makes possible the distinction of centromere-carrying micronuclei, which derive from chromosome loss, from centromere-negative micronuclei representing acentric fragments. Agents acting with a prevalent clastogenic mechanism will produce a large majority of centromere-negative micronuclei; on the contrary, aneuploidogenic agents will specifically increase the frequency of centromere-positive micronuclei. (**B**) The dual-color detection of mouse centromeric and pericentromeric sequences (tandem labeling) allows one to distinguish among three classes of micronuclei, those positive for both sequences (representing whole chromosomes), those carrying the pericentromeric satellite only (representing huge chromosome fragments deriving from breakage at the pericentromeric heterochromatic region), and the "negative" ones which represent acentric fragments; (**C**) after dual-color detection of centromeric and telomeric sequences, four categories of micronuclei are expected, which correspond respectively to segregation errors before or during anaphase, to acentric chromosome fragments, to acentric chromatid fragments.

2. Materials

2.1. Sample Preparations

1. Fully equipped cytocentrifuge (Shandon "Cytospin," UK; *see* **Note 1**).
2. Hank's balanced salt solution without calcium and magnesium (an alternative: cell culture medium without serum).
3. Fixative solution (not required for slide preparation by cytocentrifuge): 3:1 mixture of methanol:glacial acetic acid, freshly prepared. Keep on ice after preparation (*see* **Note 2**).
4. 100% Ethanol kept at –20°C.
5. Microscope slides (*see* **Note 3**).
6. Coplin jars with screwed cap.
7. Contrast phase microscope.

2.2. Single-Color Detection of Repetitive DNA Sequences in Micronuclei

1. Cell preparations, fixed on slides and maintained at –20°C.
2. Thermocycler equipped with *in situ* polymerase chain reaction (PCR) block (*see* **Note 4**).
3. The oligonucleotide corresponding to the target sequence. Synthesis of primers is conducted by several companies or facilities; oligos are stable for years at –20°C. Make aliquots to avoid repeated thawing/freezing.
4. Cocktail of dATP, dGTP, and dCTP, 5 m*M* each. Batch solutions of deoxynucleoside triphosphates must be appropriately diluted in pure (e.g., milliQ) H_2O. Store 10- to 20-µL aliquots at –20°C. The cocktail is stable for at least 1 yr.
5. dTTP, 0.5 m*M* (appropriately diluted from the batch solution in pure H_2O). Store in 10- to 20-µL aliquots at –20°C. Stable for at least 1 yr.
6. 1 m*M* Digoxigenin-11-dUTP; *see* **Note 5**), obtained from Roche Applied Science (Monza, Italy). Store at –20° C.
7. *Taq* DNA polymerase (Promega, storage buffer B, 5 U/µL; *see* **Note 6**).
8. Denaturing solution: 0.01 *M* NaOH, 1 *M* NaCl. Prepare freshly from the stock solutions (0.5 *M* NaOH, 2 *M* NaCl) and discard after use.
9. 0.01 *M* Tris-HCl, pH 7.6. Prepare from Tris-buffer, adjust pH, and autoclave. Store at 4°C and discard within 2 wk from the first utilization.
10. Stop solution: 50 m*M* NaCl, 50 m*M* ethylene diamine tetraacetic acid (EDTA). Prepare freshly from the stock solutions (2 *M* NaCl; 0.5 *M* EDTA, pH 8.0) and discard after use.
11. 20X standard saline citrate (SSC): 3 *M* sodium chloride, 0.3 *M* trisodium citrate. Store at room temperature.
12. 4X SSC, 0.1% Tween-20, pH 7.0. Dilute 100 mL of 20 X SSC in 380 mL of pure H_2O, adjust pH, and bring to 500 mL. Add 500 µL Tween-20. Keep at 4°C and discard after 2 wk.
13. Phosphate-buffered saline (PBS): 140 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, and 1.8 m*M* KH_2PO_4, pH 7.4. Autoclave and store at 4°C. Discard 2 wk after the first use.

14. PBST: PBS, 0.1% Tween-20. Store at 4°C and discard after 2 wk.
15. 10X blocking solution (Roche Applied Science). Prepare 2-mL aliquots and store at –20°C. Working aliquots can be kept at 4°C for several weeks. Discard if they become turbid.
16. Fluorescein-conjugated anti-digoxigenin mouse antibodies (Fab fragments; Roche Applied Science). Store at –20°C according to manufacturer instructions. To avoid repeated thawing/freezing, prepare 20-µL aliquots. The working aliquot can be kept for several weeks at 4°C and stored in the dark (*see* **Note 5**).
17. DAPI/antifade solution: 1.5 µg DAPI in 1 mL of Vectashield (Vector Laboratories, UK).
18. 22- × 22-mm Cover slips.
19. 24- × 24-mm Cover slips.
20. Coplin jars.
21. Nail polish (*see* **Note 7**).

2.3. Dual-Color Detection of Repetitive DNA Sequences in Micronuclei

To the list of **Subheading 2.2.**, add the following materials:

1. 1 m*M* Biotin-16-dUTP, obtained from Roche Applied Science. Store at –20°C.
2. Cy3-conjugated ExtrAvidin® (Sigma-Aldrich, Milan, Italy). Keep at 4°C according to the manufacturer's instructions, stored in the dark. Use a 1:10 working dilution for preparing the detection mix, which is a 1:300 final dilution.

3. Methods

3.1. Sample Preparations

This section describes the necessary steps to make slide preparations according to the requirements of the PRINS protocol. However the reader must refer to specific reviews on micronucleus assay ***(3,4,6)*** to define the correct experimental planning for cell treatment and harvesting (*see* **Note 8**). Slides can be prepared by hand from a cell suspension of previously fixed cells. The most efficient and recommended way to prepare slides is to use a cytocentrifuge (*see* **Note 1**).

1. A monocellular suspension must be used. When necessary, apply the appropriate digestion steps with proteolytic enzymes (*see* **Note 9**). Estimate the cell number, and then centrifuge the cell suspension. If a cytocentrifuge is available, go directly to **step 5**.
2. Discard the supernatant, and then resuspend the pellet in few drops of cool fixative solution (freshly prepared, 3:1 methanol:glacial acetic acid; *see* **Note 2**). After the pellet is completely resuspended, bring to 1.0 to 1.2 mL with fixative solution (the suspension should remain opalescent).
3. On perfectly cleaned slides (*see* **Note 3**) drop, from 5 to 10 cm of height, one to two drops of the cell suspension. Tilt immediately the slide at 45° angle, and wait for the complete fixative evaporation.

4. Check the quality of the preparation at the contrast phase microscope: cells must be evenly spaced and nonoverlapping; cytoplasm must be well preserved (*see* **Note 10**). If necessary, adjust the concentration of the cell suspension. Prepare the required number of slides and go to **step 7**.
5. For the cytocentrifugation of cells onto microscope slides, the cell density of the cell suspension is crucial. Therefore, after having discarded the supernatant, the pellet must be resuspended in the volume necessary to have approx 0.5×10^6 cells per milliliter (use only Hank's balanced salt solution [HBSS] or medium without serum; *see* **Note 11**). From this step, keep the cell suspension on ice.
6. Spot 100 µL of cell suspension (i.e., 50,000 cells) per slide, at 800 rpm, 5 min (*see* **Note 10**). Check the cell density at the contrast phase microscope: cells must be evenly spaced, not overlapping each other, with well preserved cytoplasm. Adjust if necessary the concentration of the cell suspension (*see* **Notes 10** and **12**).
7. Fill the necessary number of Coplin jars (screwed cap) with –20°C absolute ethanol and transfer the slides inside within 1 h from preparation (*see* **Note 13**). Slides can be kept at these conditions for several weeks.

3.2. Single-Color Detection of Repetitive DNA Sequences in Micronuclei

The procedure described here can be applied following the rationale depicted in **Fig. 2A**. The centromeric region of human chromosomes can be detected by means of a primer designed on the 17-base pair motif of the CENP-B box ***(7)***. This sequence is found in alphoid DNA repeats at centromeres, and it is shared by all autosomes and the X-chromosome of humans and mice ***(7)***. Therefore, the protocol described here also can be applied on murine cells (*see* **Subheading 3.3.** for a most useful protocol). With few modifications, the single-color protocol can be used for the detection of telomeric repeats in mammalian cells. In hamster, the pericentromeric regions consist of huge blocks of nonfunctional telomeric repeats ***(8)***, whereas a true centromeric probe is still not available for molecular cytogenetics. Therefore, the telomeric repeat is a marker of the pericentromeric region in hamster cell lines.

1. Immediately before starting the procedure, prepare the reaction mix as described in **Table 1** (*see* **Note 5**). Centrifuge briefly and keep on ice.
2. Move the slides from –20°C to room temperature, drain the excess ethanol, and allow to dry perfectly. Select under the phase contrast microscope the working area and mark its position on the backside of the slide with a diamond-tip pen.
3. Place a Coplin jar containing 50 mL of denaturating solution on ice, immerse the slides (maximum of four, *see* **Note 15**), and denature the preparations for 1 min, 30 s (*see* **Notes 15** and **16**). Rinse briefly in 50 mL of 0.01 *M* Tris-HCl, pH 7.6 (shake the Coplin jar gently). Transfer the slides to fresh Tris-HCl solution and incubate for 2 to 3 min to completely stop the denaturating action of NaOH.
4. Immediately before starting the PRINS reaction, add 0.5 mL of *Taq* DNA polymerase (5 U/µL; *see* **Note 6**). Prewarm the reaction mixture at the annealing temperature (**Table 2**) and proceed immediately.

Table 1
Reaction Mix for Single-Color PRINS

Mix component	Stock concentration	Final concentration	Volume (µL)
Buffer	10X	1X	2.5
$MgCl_2$	25 m*M*	1.5 m*M*	1.5
(dATP, dGTP, dCTP) cocktail	5 m*M*	0.2 m*M*	1
dTTP	0.5 m*M*	0.02 m*M*	1
Digoxigenin-11-dUTP	1 m*M*	0.02 m*M*	0.5
Primer[a]	100 µ*M*	6 µ*M*	1.5
H_2O (milliQ)			17
Total volume			25

[a]*See* **Note 14**; **Table 2**.
The quantities indicated are for two slides.

5. Remove one slide from the Coplin jar, drain the excess saline solution by keeping it briefly in a tilted position on blotting paper, and then wipe the backside. Place the slide on the prewarmed *in situ* PCR block at the annealing temperature. At this step, the cell spot must be still hydrated but not wet. Apply 12 mL of the reaction mix, cover with a 22 × 22 cover slip, and seal with nail polish (*see* **Note 7**).
6. The procedure must be repeated for all the slides as quick as possible (*see* **Note 17**), and the success of the reaction can be strongly influenced by delayed actions.
7. Start the PRINS program (**Table 2**).
8. In a Coplin jar, prewarm the stop solution to 65°C. At the end of the PRINS reaction, immerse the slides in the stop solution. After a few seconds, the nail polish seal will become soft, and can be removed easily with the help of fine forceps; generally, the cover slip detaches from the slide surface in the same moment. Otherwise, place the slide in the stop solution and shake gently: the cover slip soon after will float. Incubate the preparations for a further 2 to 3 min after the cover slips are removed.
9. Immerse slides for 5 min in approx 50 mL of 4X SSC, 0.1% Tween.
10. Transfer the slides to approx 50 mL of PBST and wash for 5 min.
11. In the meantime, the detection mix should be prepared, which is fluorescein-conjugated antidigoxigenin mouse antibody (Fab fragment), 1:12 v/v, 1X blocking solution, and PBS. For two slides, prepare 60 mL of detection mix; therefore, 5 mL of the anti-dig antibody, 6 mL of blocking solution, and 49 mL of PBS. Keep the mix on ice preserving from light.
12. Apply 30 mL of detection mix per slide, cover with a 24 × 24 cover slip, and place slides in a humidified chamber (*see* **Note 18**). Incubate slides for 1 h at 37°C.
13. Wash the slides three times (5 min each) in PBS. The cover slips will become detached in the first washing step by gently shaking the Coplin jar (*see* **Note 19**). Work in the dark as much as possible to avoid fluorescence bleaching.

Table 2
Primer Sequences and Reaction Specifications for PRINS in Micronuclei of Mammalian Cells

Primer	Sequence detected	Annealing conditions	Extension conditions
CENP-box			
5'-TGAGGCCCATCGTTGGAAAAGGAAATATC-3'	Centromeric alphoid repetitive sequences of humans and mice	53°C 10 min	63°C 30 min
Telomere			
5'-$(TTAGGG)_5$-3'	Telomeric sequences of humans and mice; pericentromeric, telomere-like sequences of hamster	53°C 10 min	72°C 30 min
Minor			
5'-GGAAAATGATAAAAACCACACTGTACAACATATTA-3'	Mouse centromeric DNA (minor satellite)	50–53°C[a] 10 min	72°C 30 min
Major			
5'-CACTTTAGGACGTGAAATATGGCGAGGAAAACTGA- 3'	Mouse pericentromeric DNA (major satellite)	50°C 10 min	63°C 30 min

[a]The size of fluorescent spots can result too small, especially in the case of tandem labeling with the large repetitive blocks of major satellite. Adjust the annealing temperature to improve the quality of the signals according to cell type and denaturation conditions.

14. Mount the slides with 25 to 30 mL of DAPI/antifade solution under a 24 × 24 cover slip.
15. Let the excess mounting medium dry. Approximately 1 h later, you can permanently seal the slides with nail polish. Slides can be maintained at 4°C until scored. Criteria for analysis and data interpretation are given in **Subheading 3.4.3.**

3.3. Dual-Color Detection of Repetitive DNA Sequences in Micronuclei

With the procedure described here, which is a two-color PRINS protocol, you can localize the centromeric and pericentromeric regions of murine chromosomes. In the mouse, the centromeric satellite DNA is called minor satellite, and the pericentromeric one major satellite, in agreement with their average extension on chromosomes. All murine chromosomes, except the Y, share the same consensus sequences, therefore specific primers can be designed ***(9)***. The possibility of labeling, in a tandem fashion, the centromeric and pericentromeric regions, offer the following advantages: (1) the presence of a whole chromosome in the observed micronucleus can be confirmed (if micronucleus is labeled with both fluorescences; **Fig. 2B**) and (2) the frequency of preferential chromosome breakages in the wide heterochromatic pericentromeric region can be investigated by estimating the frequency of micronuclei carrying the major satellite DNA only (**Fig. 2B**; *see* **Subheading 3.4.3.** for interpretation of the results).

Another powerful dual-color approach consists in the labeling of telomeric and centromeric DNA sequences in the same micronucleus (**Fig. 2C**).

1. Follow the procedure described in **Subheading 3.2.**, **steps 1** to **8**. In the meantime, prepare the second reaction mix, necessary for labeling a different repetitive DNA sequence by means of a different modified nucleotide (*see* **Note 20**) and the specific primer (**Table 2**). Calculate 25 µL for two slides.
2. Immerse the slides in a Coplin jar containing 0.01 *M* Tris-HCl, pH 7.6. Incubate for 2 to 5 min (*see* **Note 21**). You are ready for the second PRINS reaction: repeat **steps 4** to **8** described in **Subheading 3.2.**
3. The detection step is formally the same as described in **Subheading 3.2.**, **steps 9** to **15**. However, the detection mix contains both fluorescein-conjugated anti-digoxigenin antibodies (1:12 v/v) and Cy3-conjugated extravidin (1:300 v/v). For two slides (60 µL) mix 5 µL of fluoresceinated anti-digoxigenin antibody, 2 µL of a 1:10 working solution of Cy3-conjugated-extravidin (final dilution 1:300), 6 µL of blocking solution, and 47 µL of PBS.

3.4. Analysis of Preparations and Data Intepretation

As in every procedure of interphase molecular cytogenetics, labeling must be accurate to minimize the occurrence of false-positive as well as false-negative results. For example, a high frequency of centromere-negative centromeres

would indicate that the agent inducing them acts by chromosomal breakage; a poor labeling would produce false-negative results, leading to the wrong conclusions about the mechanism of action of the agent under study. After application of the protocols described here, the quality of labeling can be immediately appreciated using the fluorescence microscope. The performance of the reaction must be considered acceptable only if nuclei are evenly labeled in the working area. If fluorescent spots are faint or if labeling is uneven along the slide, the micronucleus assay cannot be conducted because the chance of false-negative results is too great. The performance of the PRINS technique is on the average high, and repeated experiments giving poor quality labeling must prompt one to analyze step by step the possible reasons of failure.

3.4.1. Selection of Cells To Be Analyzed

The scoring of acceptable slides must proceed along the working area of the slide, at ×1000 magnification; the minimum number of cells to be analyzed per replicated experimental point is 1000. However, refer to the general protocols for micronucleus assay for the recommended sample size ***(3,4,6)***. Cells must be discarded from analysis if:

a. The fluorescent spots corresponding to the sequence(s) of interest are faint.
b. The nucleus does not have a regular shape with definite boundaries.
c. Different cells are overlapping in the same area.
d. A micronucleus is found but is not clearly contained in the cytoplasm of the reference cell (*see* **Note 22**).
e. A micronucleus is found but is not clearly separate from the main nucleus (it could derive from nucleus extrusion).
f. The micronucleus and the nucleus are not on the same focus plane.
g. The diameter of the micronucleus exceeds one-third of that of the main nucleus of the cell (again the origin of the body of extra-chromatin could be different from that postulated for the micronucleus assay).
h. The shape of the micronucleus is not regular: micronuclei have a proper membrane, therefore accept only round or oval micronuclei with a clear boundary.
i. Micronucleus and nucleus stainings are of different intensities.
j. The micronucleus staining is refractile.
k. Multiple micronuclei are found: accept only cells with not more than two micronuclei to avoid including apoptotic cells in your analysis.

3.4.2. Classification of Micronuclei

The cells selected for analysis are classified, if carrying a micronucleus, with respect to the presence or absence of sequences labeled.

1. If a single-color PRINS is conducted, micronuclei can be classified as “positive” when the fluorescent spot is observed. For example, C^+MN if the micronucleus contains the centromeric fluorescent spot (*see* **Notes 23** and **24**).

2. The number of fluorescent spots visible in each micronucleus must be recorded when telomere sequences are under analysis because the number of telomeric regions expected depends on the modality of formation of the micronucleus itself (**Fig. 2C**; *see* **Notes 23** and **25)**.
3. If a dual-color PRINS procedure is conducted, the classification of micronuclei must proceed step by step in order to minimize bias classifications (*see* **Note 26**).

3.4.3. Quantitative Analysis and Discussion of Data

After microscope analysis, calculate (1) the frequency of cells carrying micronuclei with respect to the total number of cells scored. In a control cell population, expected values are in the order of 0.1–0.5%; in treated population these values may be up to ten times higher. (2) The frequency of micronucleated cells belonging to every category observed (for example: C^+MN vs C^-MN), with respect to the total number of the cells analyzed.

Use the frequencies to infer the main mechanisms of action of the agents under study (apply the appropriate statistical comparisons): agents acting with a prevalent clastogenic mechanism will produce a large number of centromere-negative micronuclei; on the contrary, aneuploidogenic agents will increase specifically the frequency of centromere-positive micronuclei. In the application of the tandem labeling approach, you can, in addition, consider the occurrence of preferential events of breakage into the pericentromeric heterochromatic region by calculating the proportion of micronuclei that are positive for the major satellite sequence on the total number of micronuclei deriving from chromosome breakage (i.e., those positive for major satellite plus negative ones). If the proportion will exceed that expected on the basis of the size of major satellite DNA (5–10%), a preferential clastogenic action on the sequence investigated must be postulated. In the dual-color labeling of centromeric and telomeric sequences, you will compare the relative frequencies of centromere-positive or -negative micronuclei to detect aneuploidogenic vs clastogenic action, and the number of telomeres will allow one to understand whether segregation errors occur more often before or during anaphase (**Fig. 2C**). Note finally that, according to the sequence(s) selected for analysis, some classes of micronuclei are not expected unless false-positive or false-negative results occurred (*see* **Note 27**).

4. Notes

1. The cytocentrifuge is particularly useful when the number of cells available is small, or for those cell types that are sensitive to the fixative treatment. Limited volumes of living cell suspensions can be sufficient to obtain perfectly flat cell preparations, which will be postfixed easily.
2. This solution is not stable and quickly loses its fixative properties.

3. The quality of slide surface is crucial for successful cell preparations and for the performance of PRINS reactions. For the best reproducibility, choose only high-quality, thin microscope slides. Although the slides are provided precleaned, it is recommended that they are washed immediately before use in detergent solution (in a hot temperature for 15–30 min). Slides are then rinsed in tap water and finally three times in distilled water. This step will be of help in keeping the background fluorescent noise as low as possible. Clean slides should be used immediately. Drop cell preparations preferentially on wet slide surfaces.
4. Different companies provide thermocyclers equipped with flat blocks, which are specifically designed for *in situ* PCR applications. A cheap alternative is the use of two thermoblocks, respectively, set at the annealing and extension temperatures. In both situations, the reproducibility of the protocol depends on how accurate the thermal profile is around the employed surface, and along the reaction time.
5. For single labeling, we recommend the use of digoxigenin-11-dUTP detected by fluoresceinated antibodies because, at least in our hands, this labeling system appears as the most efficient. Alternatively, a different modified nucleotide (e.g., biotin-16-dUTP) at the same final concentration indicated for digoxigenin-11-dUTP can be used, together with the appropriate detection protocol, as that described in **Subheading 3.3.**
6. This enzyme is strongly recommended because it gives highly reproducible results and produces sharp fluorescent spots.
7. The nail polish must be resistant to temperature because a possible pitfall is the low quality of sealing. Temperature-sensitive nail polish tends to bubble. Consequently, mix evaporation can occur, leading to failure of the reaction.
8. The micronucleus assay can be conducted with several primary cells or cell lines. The major applications of the micronucleus assay are on human, murine, or hamster cells. The only crucial requirement concerns the use of proliferating cells because the micronucleus originates at cell division. It must be reminded that the micronucleus is the ultimate consequence of a primary event which may have been occurred in a previous phase of the cell cycle. For example, it is well known that chemically-induced chromosome breakages are restricted for a large number of agents to the G_1 phase. Therefore, the experimental planning must take into account the specific cell cycle timing for the cells of interest, either in vivo or in vitro.
9. Standard trypsin–EDTA should be used for adherently growing cell lines or the recommended enzymes for dissecting cells from different tissues. Whichever protocol is used, remember that prolonged exposure to proteolytic enzymes must be avoided because it can damage cell membranes, leading to low-quality slide preparations.
10. Few trials will be sufficient to gain the experience concerning the optimal cell density required in these experiments. Analysis of low-density preparations is both time-consuming and expensive. In contrast, high-density preparations lead to extensive cell overlapping and, moreover, make it difficult to control denatur-

ation conditions along the slide area. Only Shandon Cytospin provides high-quality features for cell spotting on slides by cytocentrifugation. The Cytospin Shandon has a unique rotor and speed is only given in rpm by this manufacturer.

11. Avoid the use of PBS because the salt will deposit on the slide surface during air-drying, impairing the immediate observation of the preparation at the contrast phase microscope. If you have many cell samples to be managed at the same time or if you must work with high cell numbers, it is preferred to resuspend the cells at a higher cell density than indicated and to prepare serial dilutions immediately before use. In this way the cell integrity will remain well preserved.
12. If only a fine adjustment of the cell density is necessary, you can simply modify the volume to be spotted per slide. However, remember that cell aggregates may be formed in small volumes, whereas high volumes result in a less tight adhesion of the cells to the slide surface.
13. Some cell types are sensitive to air-drying after cytocentrifugation, which results in poor quality cytoplasm and nucleus morphology. If irregular boundaries or vacuoles are observed, it could be better to immerse the slides as soon as possible in ethanol to avoid air-drying. In this case, check for slide quality under the contrast phase microscope by keeping the slides perfectly wet.
14. According to the experimental rationale, single-color PRINS can be performed by using one of the probes listed in **Table 2**.
15. The denaturation step can be controlled more easily by handling few slides per time. The denaturation time depends on the cell type used. The time indicated is a good starting point to establish the optimal conditions. You can check under the contrast phase microscope the quality of the cells after the denaturation has been completed: denatured cells appear pale, but maintain their morphology. After PRINS, cells that are inadequately denatured will show only small fluorescent signals, and the labeling efficiency will be, on average, low (*see* **Notes 24** and **25**); the shape of fluorescent signals will appear diffused, and the nucleus morphology will be in part lost, if the denaturation was too effective. In addition, in the presence of strong denaturation conditions, small micronuclei can be completely lost. All these aspects can influence the accuracy of analysis. Remember finally that the cell density is crucial, and you will hardly obtain proper denaturation with high-density preparations.
16. For some cell types, we have found that heat denaturation can be more efficient than NaOH denaturation. For example, the highly compact chromatin of mouse spermatids and spermatozoa can be denatured at 94 to 96°C for 5 min (place 400 mL of 10 m*M* Tris-HCl, pH 7.6, on the slide and cover it with a 24 × 40-mm cover slip. Rinse for 2 min in Tris-HCl on ice). Also, depending on the preparation, a proteolytic treatment can be applied before PRINS, but it does not appear crucial for the majority of cell types.
17. To optimize the process, let a new slide drain while spotting the reaction mix on the previous one. Remember that slides must never dry completely.
18. The humidified chamber is a plastic box in which a layer of wet paper in the bottom prevents slides from evaporating, and cover slip sealing is not required.

19. If the cover slip remains tightly adhered to the slide, it is possible that evaporation of the reaction mix occurred, and in this case the labeling could be unsuccessful. Check for the quality of nail polish sealing (*see* **Note 7**).
20. For the best results, we suggest to label in the first cycle the sequence expected to do the smallest fluorescent spot. Therefore, label minor satellite DNA before major satellite, or telomeric sequences before minor satellite. Also, use the most sensitive detection system (digoxigenin/anti-digoxigenin) for the first PRINS cycle.
21. When applying a dual-color procedure and, more importantly, a tandem labeling procedure, it is crucial to avoid that the previous mix components remains available during the second PRINS reaction. A washing step after the stop solution is strongly recommended.
22. Contrast phase combined with ultraviolet light allows the accurate localization of micronuclei. However, the cytoplasmic boundaries can be detectable simply because the very faint background fluorescence.
23. Spots corresponding to either centromeric or telomeric sequences are preferentially located at the micronucleus boundary, but occasionally can be observed in inner positions. If fluorescent spots with intensity comparable to that observed into the micronuclei are visible in the cytoplasm, discard the cell from the analysis to avoid false-positives.
24. A positive control, i.e., a chemical with known aneuploidogenic activity can be used to check whether the frequency of centromere positive micronuclei is maximized as expected (**Fig. 2A**). If the frequency of centromere lacking micronuclei is still high, a false-negative result was probably obtained. Improve denaturing conditions as a possible cause for the underestimation of fluorescent signals. Micronuclei with more than one fluorescent spot are seldom observed. Record the occurrence of this event separately.
25. At least one fluorescent spot is expected in the micronuclei (**Fig. 2C**). Therefore, negative micronuclei must be considered artifacts and their frequency should be close to zero. Improve denaturing conditions as a possible cause for the underestimation of fluorescent signals.
26. Always score the fluorescent spots in the same order considered for labeling: minor satellite before major satellite sequences, telomeric sequences before minor satellite. The bias classification of micronuclei is lowest by choosing this order. Fluorescent spots corresponding to centromeric and pericentromeric signals must be juxtaposed (use the triple band filter to verify the tandem labeling). The observation of separated sequences must be recorded separately.
27. For example, you never expect micronuclei completely lacking telomeric sequences, or micronuclei carrying the minor satellite but lacking the major one. Micronuclei carrying the centromere should show at least two telomeres because they are expected to harbor a whole chromosome (**Fig. 2C**), even though a limit situation would be a micronucleus originating from the missegregation of a chromosome carrying a terminal deletion.

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10

PRINS for the Detection of Gene Deletions in Cancer

Avirachan T. Tharapel and Stephen S. Wachtel

Summary

The chromosomal regions 13q14 and 17p13 often are found rearranged in hematopoietic tumors in humans, but the rearrangements can be subtle and can escape detection on gross cytogenetic analysis. For example, submicroscopic perturbations of the *RB1* and *p53* tumor suppressor genes, located in 13q14 and 17p13, respectively, frequently occur in leukemias; this has been confirmed by molecular methods such as fluorescence *in situ* hybridization (FISH). Our group modified the primed *in situ* labeling (PRINS) method to study *RB1* and *p53* in cultured bone marrow cells from leukemia patients with known deletions within the 13q14 or 17p13 regions. Locus-specific oligonucleotide probes ("PRINS primers") were annealed to chromosomal DNA on glass slides and extended in the presence of the four trinucleotide precursors, biotin-16-dUTP, and Taq DNA polymerase. After addition of avidin-conjugated fluorophores, the resulting signals could be visualized by fluorescence microscopy in metaphase spreads and interphase nuclei of controls but were absent in the corresponding preparations from patients. The results of these and similar studies suggest that with further development, PRINS might be used as a convenient and rapid alternative to FISH in the delineation of deletions involving single genes or unique sequences.

Key Words: Gene deletions; PRINS; FISH; chromosome; cytogenetics; single-copy genes; unique sequences; leukemia; lymphoma; RB1; p53.

1. Introduction

Primed *in situ* labeling (PRINS) is a novel method that can be used as a specific and rapid alternative to fluorescence *in situ* hybridization (FISH) in the detection of subtle chromosome rearrangements ***(1,2)***. According to the PRINS method, laboratory-synthesized oligonucleotide probes ("PRINS primers") are used instead of cloned DNA probes for the *in situ* localization of individual genes or unique sequences. The primers are annealed *in situ* to complementary target sequences on slide-fixed chromosomes and extended in the presence of labeled nucleotides and *Taq* DNA polymerase ***(3)***. Chromo-

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somal DNA acts as template for the extension, and the labeled nucleotides serve as substrate for the DNA polymerase. The labeled extended sequences are visualized by fluorescence microscopy.

In its early days, PRINS was used to detect repetitive sequences such as α-satellite, *Alu*, and telomeric repeats ***(1,2,4)***. However the method now is used to identify single copy sequences—the X-linked *factor IX* gene ***(5)***, for example, and, more recently, the *SRY* sex-determining gene ***(see* ref. *6*** and Chapters 4 and 5). This shift represents a significant biological milestone because any gene with a published sequence is now amenable to study by PRINS.

After modification of the original method, our group used PRINS with the TSA™ Biotin System to evaluate *RBM* and *DAZ* gene deletions in azoospermia ***(7)***, and *SNRPN* and *GABRB3* deletions in the Prader Willi and Angelman syndromes ***(8)***. Deletion in these cases was indicated by absence of signal in experimental samples as compared with its presence in normal control subjects. Here, we describe the modified PRINS method in the study of *RB1* and *p53* gene deletions in cultured bone marrow cells from patients with hematopoietic cancers ***(9)***.

2. Materials

2.1. Bone Marrow Culture

1. RPMI-1640 culture medium (RPMI-1640; Gibco Invitrogen, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS; Invitrogen-Life Technologies, Carlsbad, CA). Store at 4°C.
2. Gentamycin sulfate (Irvine Scientific, Santa Ana, CA) as a supplement to RPMI-1640 medium. Store at 4°C and add to medium at 10 mg/mL.
3. Sodium heparin (Elkins-Sinn, Cherry Hill, NJ) as a supplement to RPMI-1640. Store at 4°C and added to cultures at 1000U/mL.
4. L-Glutamine (Gibco Invitrogen, Bethesda, MD) as a supplement to RPMI-1640. Store at –20°C and add to medium at room temperature (4 mL per 500 mL of medium).
5. KCl-Na citrate hypotonic solution (Fisher Scientific, Pittsburgh, PA): add 0.560 g of KCl to 100 mL of distilled or deionized water to make a 0.075 *M* solution. Add 0.8 g of Na citrate to 100 mL of distilled or deionized water to make a 0.8% Na citrate solution. Mix the two solutions just before use: 2:1, KCl:Na citrate. Store at room temperature and prepare a fresh mixture every 7 d.

2.2. Cell Harvest and Preparation of Slides

1. Colcemid (Gibco Invitrogen). Purchase in lyophilized form and store at 4°C. Use at room temperature after reconstitution in deionized or distilled water. Discard after 1 mo of use.
2. Glass slides and covers (Fisher Scientific).
3. Fixative solution: 3:1 methanol:glacial acetic acid (Fisher Scientific).

2.3. *Primed* In Situ *Hybridization*

1. Programmable thermal cycler equipped with a flat plate for holding slides (MISHA, Shandon Lipshaw, Pittsburgh PA; *see* **Note 1**).
2. Dimethyl sulfoxide (DMSO; Sigma Genosys, St. Louis, MO) molecular biology or high-performance liquid chromatography grade.
3. Ethanol (Fischer Scientific).
4. Oligonucleotide primers (Research Genetics, Huntsville AL; *see* **Note 2**). The following probes for the *RB1* and *p53* genes were HPLC purified, and stored at –20°C:

   RB1: 5'-TGGTAGGCTTGAGTTTGAAGA-3'
   5'-CAGCTATTTGGGAAGCTGAG-3'
   5'-TCCAGCCTGGGCAACACAG-3'
   5'-AACTTGACCAAGGTCCCTAG-3'
   5'-ACACAGCGGAATGTGTTTATG-3'

   p53: 5'-TGCTTTCCACGACGGTGA-3'
   5'-AACTGGCCAAGACCTGCCC-3'
   5'-AGCTCCTCTCCCCAGCCA-3'
   5'-ACTGTGAGGGATGTTTGGGA-3'
5. dATP, dCTP, dGTP, dTTP: from TSA™ kit; tyramide signal amplification, for chromogenic and fluorescence *in situ* hybridization and immunohistochemistry (NEN Life Science Products, Boston MA).
6. Biotin-16-2'-deoxyuridine 5'-triphosphate (dUTP; Roche Molecular Systems, Alameda, CA; formerly Boehringer-Mannheim).
7. Tris-HCl buffer (Sigma, St. Louis, MO).
8. Magnesium chloride ($MgCl_2$) (Fischer Scientific).
9. Potassium chloride (KCl; Fisher Scientific).
10. Bovine serum albumin (Invitrogen Life Technologies, Carlsbad, CA).
11. *Taq* DNA polymerase (Amplitaq, Perkin-Elmer, Foster City, CA) with TaqStart antibody (Clontech Laboraories, Palo Alto, CA).
12. 20X SSC: 3 *M* NaCl, 0.3 *M* Na citrate, pH 7.0 (Invitrogen Life Technologies).
13. Formamide–SSC (Invitrogen Life Technologies).
14. TSA Biotin System. The kit should be kept at 4°C until used, although the blocking reagent may be kept at room temperature. With proper storage, the kit is useful for 6 mo, after which the contents may become unstable.
    a. Biotinyl tyramide stock solution. Reconstitute biotinyl tyramide (amplification reagent) by addition of 0.3 to 1.2 mL of DMSO (the amount of DMSO will depend on the particular NEN kit used: 0.3 mL for 50 to 150 slides; 1.2 mL for 200–600 slides). Since DMSO freezes at 4°C, it is necessary to thaw the stock solution after removal from refrigerator.
    b. Prepare TNT washing buffer: 0.1 *M* Tris-HCl, pH 7.5, 0.15 *M* NaCl, 0.05% Tween-20. The manufacturer advises that PBS may be used as an alternative buffer, and that 0.3% Triton X-100 may be used instead of 0.05% Tween-20.
    c. TNB blocking buffer: 0.1 *M* Tris-HCl, pH 7.5, 0.15 *M* NaCl, 0.5% blocking reagent (in kit). According to the manufacturer's protocol, the blocking

reagent should be added slowly to buffer in small amounts while stirring. The mixture should be heated to 60°C with continuous stirring *(see* **Note 3**). The blocking reagent should be dissolved completely (this may take several hours). After preparation, store at –20°C.

15. Tween-20 (Sigma Genosys).
16. Triton X-100 (Sigma Genosys).

2.4. Fluorescence Microscopy and Visualization

1. Streptavidin–fluorophore conjugate (fluorescein isothiocyanate [FITC] or Texas red, also known as SpectrumRed; Vysis Inc., Downers Grove, IL).
2. Counterstain: 4',6-diamino-phenylindole (2 × 500 µL, DAPI II; Vysis Inc.).
3. Microscope equipped for fluorescence microscopy. We used an Olympus B-Max, U-M510 with triple bandpass filters, Red, Green and DAPI Bandpass, and Triple Bandpass Filter Set, DAPI/Green/Red (Vysis Inc.).
4. We used the "Color Capturing System" of Applied Imaging, Santa Clara, CA.
5. For scoring, 20 metaphase spreads were analyzed for each locus in each subject.

3. Methods

In essence, the PRINS method consists of 4 steps: (1) DNA denaturation, (2) annealing of primers, (3) chain extension in the presence of labeled nucleotides and *Taq* DNA polymerase, and (4) detection of newly-synthesized labeled DNA by use of antibodies complexed to fluorescent dyes. The oligonucleotide primers are 16 to 30 base pairs in length. A typical reaction mixture for PRINS has a volume of 40 µL and contains 200 to 500 pmol of primer *(see* **Note 4**). The reaction mixture is placed on the denatured chromosome preparation, with denaturation having been performed by immersing the slides in 70% formamide–2X SSC for 2 to 3 minat 72°C, followed by dehydration through a series of cold ethanol washes. The slides are air-dried, the reaction mixtures are layered onto the slides, and the slides are coverglassed and sealed. The slides are put on a flat-plate programmable thermal cycler (a water bath can be used instead) and maintained at annealing temperatures for 5 to 10 min, followed by extension for 20 to 30 min at 70–72°C. The reaction is stopped by immersing the slides in blocking solution (500 m*M* NaCl, 50 m*M* ethylene diamine tetraacetic acid, pH 8.0) for 5 min at 72°C. Labeled DNA is detected by immunocytochemistry and fluorescence microscopy. In our studies, biotin-complexed fragments are visualized by use of fluoresceinated avidin. The chromosome preparations are counterstained with DAPI and scored under ultraviolet (UV) light. The specific step-by-step procedures are listed in the subheadings to follow.

3.1. Preparing the Culture Medium

1. Mix 100 mL of fetal bovine serum (FBS) with 400 mL of RPMI-1640 in a separate bottle. Agitate well. Prepare fresh mixture every 30 d. Store at 4°C.
2. To each bottle of medium, add 3 mL of sodium heparin, 2 mL of gentamycin sulfate, and 4 mL of L-glutamine.

3.2. Initiation of Bone Marrow Culture

1. Two cultures are initiated for each patient.
2. For each culture, add 10 mL of culture medium to two 15-mL centrifuge tubes.
3. Using a Pasteur pipet, inoculate each centrifuge tube with 12 to 15 drops of cultured bone marrow cells. Each centrifuge tube represents a separate culture. Do not add mitogen. Label the tubes.
4. Incubate the cultures at 37°C for 72 h (in general the cultures may remain undisturbed for the full incubation period, although some groups gently agitate the cultures each day).

3.3. Harvesting the Cells

1. Using an 18-gage syringe needle, add five drops of colcemid (10 μg/mL) to each culture.
2. After mixing well, incubate the cultures for 1 to 2 h at 37°C. It may be necessary to increase incubation time with colcemid.
3. Centrifuge at 250*g* for 10 min.
4. Remove the supernatant and break up the pellet by agitation with a vortex mixer.
5. Add 10 mL of hypotonic KCl-Na citrate mixture to each culture. Suspend the cells by gently inverting the centrifuge tubes manually. Allow the tubes to stand at room temperature for 30 min.
6. Suspend the cells again by gently inverting the tubes. Then, add 2 mL of freshly prepared fixative solution directly to each tube. Mix the contents by inverting the tubes, and centrifuge the tubes again at 250*g* for 10 min.
7. Remove all of the supernatant from each culture. Gently "thump" the tubes to break up the cellular pellets.
8. Add 10 mL of fresh fixative to each culture. Resuspend the cell pellets by inverting the tubes and allow the cultures to stand at room temperature for 10 min and centrifuge.
9. Repeat the preceding step.
10. The next steps are performed in a "harvesting room" with a humidifier. For maximal chromosome spreading, adjust the humidity to 55 to 65%.
11. Set a hot plate to 65°C and check the temperature with a surface thermometer.
12. Centrifuge the cell suspension at 250*g* for 10 min.
13. Remove the supernatant from the centrifuge tubes and add methanol:glacial acetic acid fixative to the pellet drop by drop until the suspension becomes semiclear. The amount of fixative required will depend on the size of the pellet. The final cell concentration may have to be adjusted after evaluation of the first slide.

3.4. Preparing Slides for PRINS

1. Clean microscope slides are placed in slide tray containing deionized water. The slides are chilled in a refrigerator.
2. One cold slide at a time is removed from the slide tray and maintained in a slanting position on a stand or a device (at approximately a 10° to 20° angle).
3. Drop or place 40 to 50 µL of cell suspension on the slide. Allow the cell suspension to roll down the slide. Wipe the back of each slide and shake off excess fixative and water. Label the slides and place on a hot plate at 65°C for 2 to 3 min.
4. Store slides at room temperature for 24 h, after which the chromosomes are ready for the labeling procedure *(see* **Note 5**).

3.5. PRINS Labeling: Standard Protocol

1. Immerse slides in 0.02 *N* HCl for 20 min *(see* **Note 6**).
2. Immerse slides in 70% formamide–SSC, pH 7.0, for 2 min at 72°C to denature chromosomal DNA.
3. Dehydrate slides by passage through an ice-cold ethanol series, 70%, 85%, and100% EtOH, 5 min each. Air-dry.
4. Prepare reaction mixture in a final volume of 40 µL containing 50 pmol of each oligonucleotide primer (*see* **Note 7**), 0.2 m*M* each dATP, dCTP, and dGTP, 0.02 m*M* dTTP, 0.02 m*M* biotin-16-dUTP, 50 m*M* KCl, 10 m*M* Tris-HCl, pH 9.0, 2 m*M* $MgCl_2$, 0.01% BSA, and 2 U *Taq* DNA polymerase with TaqStart antibody (*see* **Note 8**). Pipet 40 µL of reaction mixture onto freshly prepared slide (*see* **Note 9**).
5. Cover the working area of the slide completely with a cover glass. Apply a thin application of rubber cement to seal the ends of the cover glass.
6. Incubate slides. Our incubations are carried out on a programmable thermal cycler equipped with a flat plate for slides (MISHA, Shandon Lipshaw; *see* **Note 1**). The program consists of one cycle of 10 min at annealing temperature with an additional 30 min at 72°C for extension. In the original study by Tharapel and Kadandale ***(9)***, 62.8°C was used for *RB1* and 64.5°C for *p53* (*see* **Note 10** for general computation of annealing temperatures).
7. After extension, slides are removed from cycler, the cover glasses are removed, and the slides washed in 0.4X SSC at 72°C for 2 min to stop the reaction (*see* **Note 11**).
8. In our studies, biotin-labeled nucleotides are detected with the TSA Biotin System.

3.6. Signal Amplification Using the TSA Biotin System

1. For each test, dilute the stock solution of biotinyl tyramide 1:50 with 1X amplification diluent to prepare the working solution; 100 to 300 µL of working solution is needed for each slide.
2. After hybridization, block slides by incubation with 100 to 300 µL of TNB buffer. This may be performed for 30 min in the humidified chamber of the cycler with

biotin-labeled probes: 100 to 300 µL of SA-HRP (streptavidin-horseradish peroxidase from the TSA kit) diluted 1:100 in TNB buffer. Fluorescein-labeled probes may be substituted: 100 to 300 µL of anti-fluorescein-HRP (NEN) diluted 1:250 in TNB buffer. Optimal concentrations of HRP-labeled reagents should be determined for individual laboratories.
3. Wash slides in TNT buffer with agitation three times for 5 min at room temperature.
4. Pipet 100 to 300 µL of working solution onto each slide. Maintain slides at room temperature for 5 to 10 min.
4. Repeat washing step (i.e., **step 3**).

3.7. Fluorescence Microscopy and Visualization

1. To each slide, add 100 to 300 µL of streptavidin–fluorophore conjugate diluted in TNB buffer; use dilution recommended by manufacturer: streptavidin-Texas red (NEN) is used at 1:500.
2. Place slides in a humidified chamber for 30 min at room temperature.
3. Wash slides in TNT buffer, with agitation three times for 5 min at room temperature.
4. Place two drops of 4',6-diamino-phenylindole (DAPI II) on the slide for chromosome staining, and mount for microscopy. Blot excess DAPI, cover, and seal the ends of the cover glass with rubber cement. Refrigerate at 4°C for 30 to 60 min (*see* **Notes 12** and **13**).

4. Notes

1. An alternative programmable cycler, the "HYBrite Denaturation/Hybridization System," is produced by Vysis Inc. However, annealing and extension can be accomplished on thermoblocks, or even in metal containers suspended in hot water baths ***(3)***. As always temperature is critical with PRINS and must be carefully controlled.
2. In our experience, signal is increased by use of multiple primers (as many as four or five) for a single locus and by single-step annealing and extension.
3. Innis and Gelfand ***(10)*** note that at 20°C, Tris buffer has a pK_a of 8.3, and $\Delta\ pK_a$ of –0.021/°C. Therefore, the actual pH of Tris-HCl may vary during thermal cycling.
4. An unamplified slide without TSAG reagents and an amplified slide without primer should be included as controls for each hybridization.
5. Slides should be kept moist during the PRINS procedure. If a humidified chamber is not available, cover slides with a damp paper towel in a closed box. If a humidifier is available, maintain humidity at 55 to 65% for optimal chromosome spreading.
6. Treatment of slides with 0.02 *N* HCl removes loosely bound protein thereby rendering DNA more accessible to the primers.
7. For each study, the primer concentration should be optimized. New England Nuclear recommends a 10-fold reduction in "probe" (primer) concentration as

optimal. This is a critically important step in PRINS, as improper concentration of probe can obviate development of the hybridization signal.

8. TaqStart monoclonal antibody binds *Taq* DNA polymerase, thereby minimizing nonspecific amplification and formation of primer dimers.
9. Reagents should completely cover cells or metaphase spreads on microscope slides.
10. After counting the A, C, G, and T nucleotide residues of the primers, annealing temperatures are computed by use of either of the following formulas:

$$T_M = 69.3 + 0.41 (\% G + C) – 650 / L$$

where L = the length of the primer = the total number of nucleotides in the primer.

$$T_M = 4 (G + C) + 2 (A + T)$$

When different temperatures are obtained, the results may be averaged. In general, satisfactory annealing occurs at temperatures between 55° and 75°C. The higher temperatures increase annealing specificity.

11. Background staining is minimized by stringent washing of slides in SSC.
12. Low signal may be corrected by titration of HRP conjugate to optimize concentration, by increasing incubation time or concentration of amplification reagent, or by addition of a step to optimize penetration of reagents.
13. Background staining may be minimized by decreasing concentration of HRP conjugate or primers, by increasing endogenous peroxide quenching, by filtration of buffers, or by increasing number of washes or the length of washes.

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11

Combined Fluorescence *In Situ* Hybridization and PRINS for the Analysis of the *Dystrophin* Gene

Liborio Stuppia, Dario La Sala, and Caterina Cinti

Summary

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy are caused in most cases by deletions of the *DMD* gene. These rearrangements are detectable in affected boys and men by a simple multiplex polymerase chain reaction approach. However, this technique is not able to disclose *DMD* deletions in heterozygous female carriers, and different approaches must be used in these cases. Here, we describe an approach based on the combined use of primed *in situ* labeling and fluorescence *in situ* hybridization techniques for the detection of single DMD exons in fixed metaphase chromosomes and interphase nuclei of both male and female subjects, suggesting the usefulness of this tool in the detection of small intragenic deletions of the *DMD* gene.

Key Words: PRINS; FISH; muscular dystrophy; *in situ* single-copy gene detection.

1. Introduction

In the last 20 yr, the positional cloning approach has allowed the identification of the majority of the genes involved in the most important human genetic diseases. Studies conducted on large samples of affected patients have disclosed specific mutations associated to the diseases, allowing us to identify specific genotype–phenotype correlations. Consequently, screening of mutations of these genes is now conducted to confirm clinical diagnosis or to identify healthy carriers of genetic diseases.

So far, several techniques have been developed that are capable of performing large-scale screening of gene point mutations; however, the search for gene deletions still poses a challenge in diagnostic laboratories. In fact, although deletions of a few base pairs are easily detectable by polymerase chain reaction (PCR) amplification and DNA sequencing, and very large deletions are evidenced by cytogenetic investigations, great difficulties arise in detecting deletions of a few kilobases, which can be missed by both techniques ***(1)***. Because

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several single-gene disorders are caused mainly by deletions or duplications of part or all the gene (e.g., Duchenne muscular dystrophy [DMD], spinal muscular atrophy, Charcot-Marie-Tooth disease, Fanconi anemia, and others), diagnostic analysis of these diseases can be time-consuming and often inconclusive. Among these, DMD is of particular relevance because of the frequency and severity of the disease. DMD (MIM #310200) and Becker muscular dystrophy (MIM #300376) are the most common form of dystrophinopathy, with a reported incidence of 1:3500 and 1:18000 per birth males, respectively. These diseases are due to mutations of the *DMD* gene (Xp21.2 ***[2]***). *DMD*, which produces the protein dystrophin, is the largest human gene known so far, spanning 2.4 mb on the human genome, containing 79 exons, and encoding a 14-kb messenger RNA ***(3)***. Both diseases show progressive symmetrical muscular weakness, with DMD characterized by an earlier onset and a more severe progression. Approximately 98% of DMD deletions are easily detectable in affected men and boys using a multiplex PCR approach able to simultaneously amplify exons mapped within two hot-spot regions (exons 2–20 and 44–53 ***[4–6]***). Because no effective treatment is so far available for these diseases, the identification of carrier females is critical to prevent the birth of affected males. However, the multiplex PCR approach is not able to detect heterozygous female carriers because, in these cases, the normal X chromosome masks the presence of deletions. Different strategies must thus be used for the identification of female carriers.

In the last two decades, fluorescence *in situ* hybridization (FISH) analysis has represented a reliable and powerful tool in physical gene mapping and in the detection of gene rearrangements such as deletions, translocations, and duplications. The increased availability of specific probes for the most relevant human genes and the use of fluorescence instead of isotopic elements for the detection of the *in situ* hybridization have made this technique as a tool of choice in the molecular diagnosis of several genetic diseases. However, the typical FISH approach still has some limits. In fact, small gene rearrangements, such as deletions involving one or few exons, cannot be evidenced by this technique. Moreover, FISH analysis remains a low throughput analysis as compared with molecular genetics techniques.

Consequently, great efforts have been made to develop new strategies able to combine the benefits of cytogenetic analysis with those provided by the use of molecular techniques. The primed *in situ* labeling (PRINS) technique is based on the specific hybridization of a short unlabeled olinucleotide (approx 20–30 pb) with a denaturated template and synthesis of a single strand *in situ*, catalysed by a *Taq* polymerase. During primer elongation, labeled nucleotides are incorporated into the newly synthesized DNA, allowing their detection by

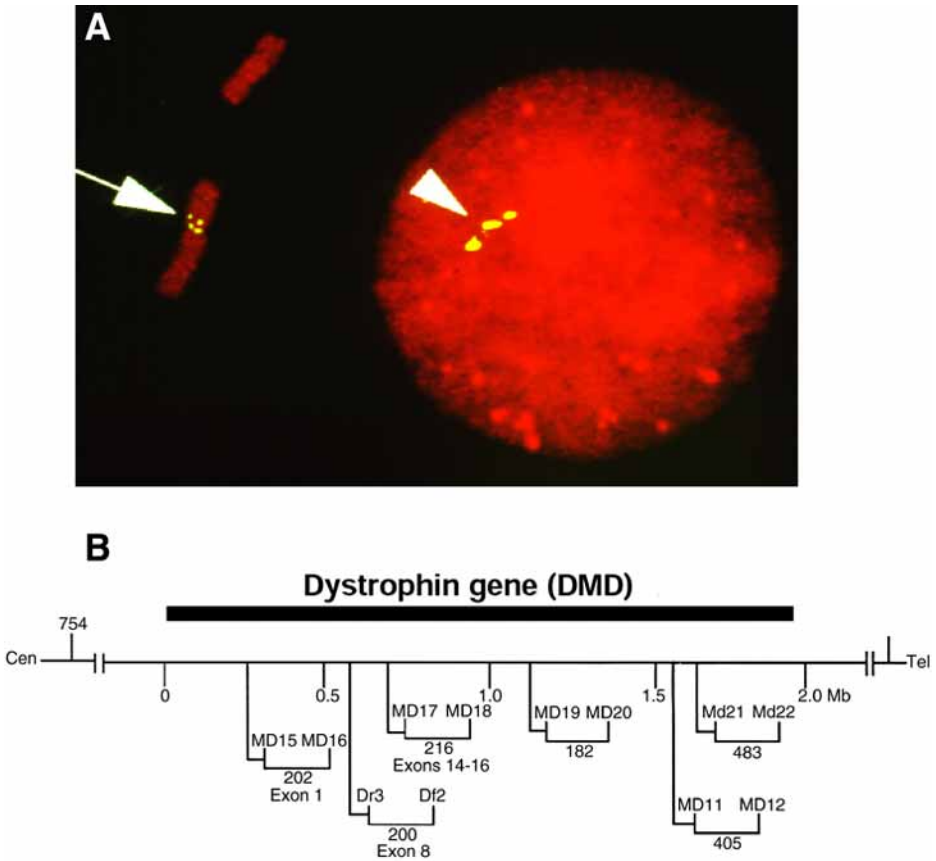

Fig. 1. (**A**) Example of *DMD* gene and X centromer labeling in chromosome and nucleus using PRINS and FISH protocol. Two oligonucleoties for exon 1 *dystrophin* gene: MD15 5'-TCTGGGAGG CAATTACCT TC-3' and MD16 5'-ACAGTCCTCT ACTTCCC-3', have been used. The image was obtained by using confocal laser microscopy. (**B**) *DMD* gene: position of exons and primers respect to X centromer (Cen ***[7,8]***). (Please *see* color insert following p. 48.)

means of specific fluorescent antibodies. This method is able to produce multiple independent signals within the same gene segment, thus allowing the detection of partial deletions within a single gene.

In this chapter, we describe the use of the combined PRINS and FISH methods for the *in situ* detection of single DMD exon in fixed metaphase chromosomes and interphase nuclei (**Fig. 1A**). This approach could allow the identification of female carries of small intragenic deletions of the *DMD* gene.

2. Materials

2.1. Preparation of Metaphase Spreads

2.1.1. Preparation From Whole Peripheral Blood Cells

1. Histopaque-1077 Lymphocyte separation medium (Sigma-Aldrich, St. Louis, MO).
2. Peripheral blood karyotyping medium (Gibco/BRL, Bethesda, MD; *see* **Note 1)**.
3. Phosphate-buffered saline (PBS): Prepare 10X stock with 1.37 *M* NaCl, 27 m*M* KCl, 100 m*M* Na_2HPO_4, and 18 m*M* KH_2PO_4 (adjust to pH 7.4 with HCl if necessary). Prepare working solution by dilution of one part with nine parts distilled water and filtration with a 0.2-µ*M* filter.

2.1.2. Preparation From Cultured Cells

1. Appropriate culture medium, supplemented with 10% fetal bovine serum (Gibco/BRL) containing 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco/BRL).
2. Solution of trypsin (0.25%) and EDTA (1 m*M*; Gibco/BRL).
3. 1X Sterile PBS.

2.2. Preparation of Metaphase Chromosomes

1. Colcemid® Solution, liquid (10 µg/mL), in 1X PBS (Gibco/BRL; *see* **Notes 2** and **3**).
2. Hypotonic solution: 0.075 *M* potassium chloride (*see* **Note 2**).
3. Fixative solution: mix precooled (–20°C) absolute methanol (Sigma-Aldrich) and glacial acetic acid (Sigma-Aldrich) in a 3:1 ratio (v/v).
4. Ethanol solutions: 100%, 90%, and 70% ethanol.

2.3. PRINS Reaction

1. Formamide solution: 70% deionized formamide, 10% 20X standard saline citrate (SSC), 10% 0,1 *M* Sorensen buffer, pH 7.4, 10% distilled water (*see* **Note 4)**.
2. 20X SSC solution: 0.3 *M* sodium citrate trihydrate, 3 *M* NaCl, adjust to pH 7.0 with citric acid. 2X SSC solution is prepared by dilution of one part of 20X SSC with nine parts distilled water.
3. 0.1 *M* Sorensen buffer: 12.0 m*M* NaH_2PO_4, 69.0 m*M* Na_2HPO_4, adjust to pH 7.4 with HCl.
4. PRINS-modified mixture: 1X PCR buffer with 1.5 m*M* $MgCl_2$ (QIAGEN, Valencia, CA), 0.5 U/µL *Taq* DNA polymerase (QIAGEN); DIG-dNTP labeling mix (40 µ*M* each of 2'-deoxyadenosine 5'-triphosphate, 2'-deoxycytosine 5'-triphosphate, 2'-deoxyguanosine 5'-triphosphate and 14 µ*M* DIG–2'-deoxyuridine 5'-triphosphate, 26 µ*M* 2'-deoxythymidine 5'-triphosphate in 5% glycerol), 4 µ*M* oligonucleotide primers, that is, for exon 1 *dystrophin* gene (MD15 5'-TCTGGGAGG CAATTACCT TC-3' and MD16 5'-ACAGTCCTCTAC TTCCC-3'; **Fig. 1B *[7,8]***), 2 ng/mL human α-satellite chromosome X-specific DNA probe, digoxigenin labeled (Q-Biogene, Irvine, CA; *see* **Notes 5** and **6)**.
5. Stop solution: 50 m*M* NaCl, 50 m*M* EDTA, pH 8.0 (*see* **Note 7)**.
6. Washing buffer: 0.05% Triton X-100 in 4X SSC (*see* **Note 8**).

7. Blocking solution: 0.5% bovine serum albumin (Sigma-Aldrich) in 4X SSC, pH 7.0.
8. Antidigoxigenin antibody: monoclonal antidigoxigenin-FITC (Roche Molecular Biochemicals, Mannheim, Germany) dilute 1:100 in 4X SSC, 0.5% bovine serum albumin, pH 7.0 (*see* **Note 9**).
9. Propidium iodide solution: 1 µg/mL propidium iodide (PI; Sigma-Aldrich) in distilled water.
10. Antifading reagent-DABCO, 2% (w/v) in 50% glycerol/1X PBS.

3. Methods

3.1. Preparation of Metaphase Spreads

3.1.1. Preparation From Whole Peripheral Blood Cells

1. Mix 5 mL of whole blood with 5 mL of 1X PBS.
2. Provide 7.5 mL of separation medium and overlay carefully with whole blood/1X PBS mixture and centrifuge for 30 min at 950*g*.
3. With a Pasteur pipet, remove carefully the portion containing the interphase lymphocytes (white ring) and wash with 50 mL of 1X PBS.
4. Centrifuge for 10 min at 400*g* and remove the supernatant.
5. Suspend the cells in 5 mL of karyotyping medium (approx 10^6 cells/mL medium).
6. Incubate 48–72 h at 37°C and 5% CO_2.

3.1.2. Preparation From Cultured Cells

These steps describe the treatment of adherent cells. Cells grow as suspension does not need the trypsin/EDTA treatment. In this case the method is aimed for 10 mL of cell suspension.

1. Remove the medium from a 75-cm^2 culture flask.
2. Wash with 10 mL of 1X PBS.
3. Incubate at 37°C with 1 mL of trypsin–EDTA solution.
4. Add 9 mL of medium, harvest the solution, and centrifuge at 150*g* for 10 min.
5. Deplete the supernatant and wash the pellet with 1X PBS.
6. Resuspend cells in karyotyping medium at concentration of 10^6 cells/mL medium.
7. Incubate 24 to 48 h at 37°C and 5% CO_2.

3.2. Preparation of Metaphase Chromosomes

Metaphase chromosomes were freshly prepared by using a spindle inhibitor such as Colcemid to arrest cultured cells during mitosis (*see* **Notes 2** and **3**).

1. Incubate 5 mL of cells suspension with 0.6 µg/mL Colcemid for 3 h at 37°C and 5% CO_2.
2. Remove the cell suspension containing the Colcemid from the incubator and mix gently. Centrifuge for 6 min at 150*g*.
3. Remove the supernatant with a Pasteur pipet.

4. Add 1 mL of warmed hypotonic solution to the tube (*see* **Note 2**).
5. Mix gently.
6. Add 9 mL of hypotonic solution (*see* **Note 10**).
7. Incubate at 37°C for 15 to 20 min.
8. Centrifuge for 6 min at 150*g*.
9. Remove the supernatant.
10. Add drop by drop 5 mL of ice-cold fixative solution to the centrifuge tube (*see* **Note 11**).
11. Pipet with a Pasteur pipet to resuspend the pellet and mix the fixative.
12. Incubate this suspension at –20°C for 30 min (*see* **Note 12**).
13. Centrifuge for 6 min at 150*g*.
14. Remove the supernatant.
15. Add 6 mL of cold fixative and mix to resuspend the pellet. Centrifuge for 6 min at 150*g*.
16. Repeat **steps 14** and **15**.
17. Finally, the chromosome suspension is spread onto cleaned slide. Withdraw the cell suspension into a Pasteur pipet. From a height of approx 30 cm, drop two or three drops of fluid onto a clean slide.
18. Allow the slides to dry thoroughly (37°C, overnight).
19. Chromosomes are dehydrated in an ethanol series (70%, 90%, and 100%) for 2 min per step and are air-dried.
20. Slides can then be stored at room temperature until use in PRINS and FISH reactions.

3.3. PRINS Reaction

1. Denature the samples by immersing them in the formamide solution (*see* **Note 4**) at 72°C for 2 min and then dehydrate them in a series (70%, 90%, and 100%) of ice-cold ethanol washes (4°C) before allowing them to air-dry.
2. Cover each sample with 50 µL of PRINS-modified mixture and top with a cover slide, which is sealed with rubber cement glue. Denature the target DNA at 95°C for 5 min using a hot plate or a thermostat protein-free water bath.
3. Transfer the slides in a hot plate or in a prewarmed water bath at 58°C for 20 min (*see* **Note 13**).
4. To perform the elongation step, transfer the slides in a hot plate or in a prewarmed water bath at 72°C for 40 min (*see* **Note 13**).
5. Remove the cover slide.
6. Stop the hybridization/elongation reaction by washing the slides with the stop solution containing NaCl and EDTA for 2 min at 60°C.
7. Wash twice with the washing buffer for 5 min per wash at room temperature to remove excess reaction mixture. Place the slides in an orbital shaker and shake during the washing.
8. Place the slides in a blocking solution for 10 min at room temperature to minimize nonspecific signals.

9. Incubate the slides with a solution containing fluorescein-isothiocyanate (FITC)-conjugated antidigoxigenin antibody for 30 min at room temperature in a dark humid chamber.
10. Wash the slides twice with washing buffer for 5 min at room temperature to remove excess antibody. Shake during the washes.
11. To stain the unlabeled DNA, incubate the slides with a PI solution for 4 min in the dark room at room temperature and then wash for 2 to 3 min under running water.
12. Allow to air-dry in the dark.
13. Apply 20 µL of antifading solution on cover slide (24 × 24 mm).
14. Cover each slide with a cover slide. For longer storage and to prevent the cover slide from sliding, the edges of the cover slide can be sealed with nail polish and conserved in the dark at –20°C.
15. Observe slides by immunofluorescence or confocal laser microscopy (*see* **Note 14**).
16. Record digitally.

4. Notes

1. Karyotyping medium is based on RPMI-1640 basal medium, supplemented with L-glutamine, fetal bovine serum, and antibiotics (penicillin and streptomycin) and containing phytohemagglutinin.
2. Prewarm the Colcemid and the hypotonic solution in the incubator at 37°C.
3. **Caution**: Colcemid can be dangerous, so handle with care. Colcemid is a mitotic spindle inhibitor. If splashed on skin, rinse immediately and seek medical help.
4. **Caution**: The formamide is toxic and may cause harm to the unborn children. Harmful by inhalation, by contact with skin, or if swallowed. Therefore, the formamide solution needs to be prepared and used by taking precautionary measures for safety work.
5. The primers used to amplify *in situ*-specific exons of *DMD* gene need to be of 20 to 22 bp and have the annealing temperature ranging from 55 to 58°C.
6. All reagents are stable at –20°C. Repeated freezing and thawing should be avoided. The PRINS-modified mixture must be freshly prepared before each use.
7. To prepare 0.5 *M* EDTA, pH 8.0, dissolve 186.1 g of EDTA disodium salt in 800 mL of distilled water and mix on magnetic stirrer using slight heating. Adjust the pH at room temperature and then add distilled water to final volume of 1000 mL.
8. Triton X-100 is a dense liquid; therefore, a tip with large lumen need to be used. Mix and dissolve completely by using magnetic stirrer.
9. For FITC-conjugated antidigoxigenin antibody, the reconstituted solution is stable at –20°C. To avoid repeated freezing and thawing, the solution must be stored in aliquots at –20°C protected from light.
10. The hypotonic solution should not be in contact with the cells for more than a total of 27 min. Excess exposure may cause rupture of the white blood cells.
11. The fixative solution must be made fresh.

12. Thirty minutes is a minimum. It is possible to keep samples in the refrigerator overnight.
13. If the annealing and elongation reactions are performed using hot plate, it is necessary to increase the temperature setting of 2°C to avoid the dispersion of temperature on glass.
14. For the acquisition of images by confocal laser microscopy, FITC and PI need to be excited with the blue (488 nm) and green (514 nm) line of the argon ion laser, respectively. Thereafter, serial optical section of FITC signal, performed on *z*-axis and merged with the corresponding PI images, will be elaborated and reconstructed as a three-dimensional projection.

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12

Brain Tissue Preparations for Chromosomal PRINS Labeling

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Summary

The preparation of a somatic cell tissue is a key point of any successful molecular biology and genetic analysis of chromosome complement and genome variations. Current molecular biology investigations require an increased amount of different tissues to study. The genetic studies of the brain became an intriguing part of oncology, neurogenetics, and psychiatric genetics. The brain tissue processing for molecular cytogenetic analysis has special peculiarities quite different from the protocols used in the field. Despite some pilot cytogenetic studies of chromosome complement in mammalian brain, the specified protocol of the brain sample preparation for molecular cytogenetic analysis has not been thoroughly described. Here, we propose the detailed brain tissue processing protocol that could be used for different molecular cytogenetic approaches to study chromosome complement in interphase nuclei.

Key Words: Developing brain tissues; cultured fetal brain tissues; frozen brain tissues; paraffin sections of brain tissues; PRINS.

1. Introduction

The studies of mammalian brain are an intriguing part of molecular biology. The growing number of investigations of mechanisms implicated in brain development, function, and disease has brought new insights into our understanding of the mammalian nervous system organization. Pilot molecular cytogenetic studies have indicated the occurrence of chromosomal variations manifested as aneuploidy in the developing and adult mammalian brain ***(1–4)***.

These findings lead to suggest that in coming years the molecular cytogenetic analysis of brain tissues is going to be an important part of genome biology ***(4,5)***. The molecular cytogenetic analysis of different types of tissues often is complicated by misinterpretation of the results obtained. This is caused by in

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large part neglecting tissue preparation specifications. Therefore, the detailed protocol of the most complicated tissues to prepare is of importance. The complicity of cultivating the majority of brain tissues has lead us to consider the interphase nuclei investigation of chromosome complement as a most appropriate way to conduct molecular cytogenetic analysis. To present the well-reproducing protocol of brain tissue processing for molecular cytogenetic analyses of chromosome complement in interphase nuclei, we have complied together the experience of different tissue preparations, such as developing brain, cultured fetal brain, frozen brain tissues, formalin-fixed brain tissue, and paraffin sections of brain tissues.

In addition to different brain tissue-processing protocols, we also have described the quality control procedure because it seems for us to be an important part of the successful molecular cytogenetic analysis. The quality control of the tissue preparation is the final step before the proceeding to primed *in situ* labeling (PRINS) (or fluorescence *in situ* hybridization) analysis. This procedure allows excluding the usefulness specimens not to make an unproductive use of molecular cytogenetic probes. There are two possibilities of control to be conducted. The visualization of suspension obtained by means of light microscope equipped by phase contrast optics provides by the information about the concentration and distribution of nuclei in cytogenetic slide preparation. In addition, the DAPI staining of nuclei allows assessing the quality of the suspension obtained and the level of autofluorescence by means of fluorescent microscope. However, it should be noted that light microscope visualization and DAPI staining are more effective when used together.

2. Materials

2.1. Preparation of Interphase Nuclei Suspension From the Specimens of Fetal and Adult Brain Tissues

1. Taped homogenizer: Teflon pestle and glass tube (Cole-Parmer International; cat. nos. A-04368-32 for Teflon pestle and A-04368-33 for glass tube).
2. Earle's buffered saline solution (EBBS; cat. no. 24010-035, Gibco Invitrogene, SARL; Cergy Pontois Cedex, France).
3. Solution of phosphate-buffered saline (PBS) pH 7.3, containing 0.1% (w/v) of Nonidet P-40. PBS preparation: prepare 10X stock water solution with 1.37 *M* NaCl, 27 m*M* KCl, 100 m*M* Na_2HPO_4, and 18 m*M* KH_2PO_4 (pH 7.4 is adjusted by 1 *N* HCl). The solution is stored at room temperature.
4. 60% (w/v) solution of glacial acetic acid.
5. Fixative mixture of methanol:glacial acetic acid (3:1, v:v) freshly prepared and stored at –20°C. **Caution:** methanol is extremely toxic (*see* **Note 1**).
6. Ethanol 100% and 96%, and water-diluted ethanol to 70%.

7. Pepsin solution: 10% (w/v) pepsin solution (stored at –20°C) is diluted in prewarmed (37°C) solution of 0.01 *N* HCl (chlorohydric acid). Pepsin solution is made fresh as required.
8. Coplin jar (50 mL).
9. Microscope slides, 25 × 75 × 1 mm, plain.

2.2. Preparation of Cell Suspension From Cultured Fetal Neuronal Cells (Short-Term or Long-Term Fetal Brain Cultures and Neuronal Stem Cell Cultures)

1. Hypotonic solution (0.9% NaCl water dissolved).
2. EBBS (cat. no. 24010-035, Gibco Invitrogene).
3. Fixative mixture of methanol:glacial acetic acid (3:1, v:v) freshly prepared and stored at –20°C. **Caution:** methanol is extremely toxic.
4. Ethanol 100%, 96%, and water-diluted ethanol to 70%.
5. Pepsin solution: 10% (w/v) pepsin solution (stored at –20°C) is diluted in prewarmed (37°C) solution of 0.01 *N* HCl (chlorohydric acid). Pepsin solution is made fresh as required.
6. Coplin jar (50 mL).
7. Microscope slides, 25 × 75 × 1 mm, plain.

2.3. The Preparation of Formalin-Fixed and Paraffin-Embedded Sections of Brain

1. Ethanol 100%, 96%, and water-diluted ethanol to 70%.
2. 20X Standard saline citrate (SSC): 3 *M* sodium chloride, 0.3 *M* trisodium citrate. The solution is stored at room temperature. The working solution is prepared by dissolving one volume of 20X SSC in four volumes of water and adding Tween-20 to 0.5% (*see* **Note 2**).
3. Pepsin solution: 10% (w/v) pepsin solution (stored at –20°C) is diluted in prewarmed (37°C) solution of 0.01 *N* HCl. Pepsin solution is made fresh as required.
4. PBS solution.
5. PBS/$MgCl_2$ working solution: 1 volume of 2 *M* $MgCl_2$ in 38 volumes of 1X PBS.
6. Formaldehyde/PBS/$MgCl_2$ solution: Add 2.7 mL of formaldehyde (37%) in 100 mL of PBS/$MgCl_2$ solution (to produce 1% of formaldehyde in PBS/$MgCl_2$ working solution).
7. RNase (0.5% w/v).
8. Sodium isothiocyanate (NaSCN) 1 *M* (w/v) for disruption of DNA–protein complexes. **Caution:** NaSCN is toxic; the wearing of gloves is indispensable.
9. Deparaffinization solvent: Xylene (100%).
10. Coplin jar (50 mL).
11. Microscope slides, 25 × 75 × 1 mm, plain.

2.4. Quality Control

1. Microscope slides, 25 × 75 × 1 mm, plain.
2. 24- × 24-mm Cover slips (CML France, Nemours Cedex, France).
3. Ethanol 100% and 96%.
4. Nuclear stain: 300 n*M* DAPI in water.
5. Sudan black ethanol–water solution. Dissolve 0.7 g of Sudan black in 100 mL of 96% ethanol, then add and stir with 50 mL of water. The solution is stored at room temperature.
6. Coplin jar (50 mL).

2.5. Multicolor PRINS

1. dATP: 100 m*M* solution (Roche Diagnostics, Meylan, France) diluted 1:10 with sterile distilled H_2O.
2. dCTP: 100 m*M* solution (Roche Diagnostics) diluted 1:10 with sterile distilled H_2O.
3. dGTP: 100 m*M* solution (Roche Diagnostics) diluted 1:10 with sterile distilled H_2O.
4. dTTP: 100 m*M* solution (Roche Diagnostics) diluted 1:100 with sterile distilled H_2O.
5. Fluorescein isothiocyanate 2'-deoxyuridine 5'-triphosphate (FITC-12-dUTP): 1 m*M* (Roche Diagnostics).
6. Tetramethylrhodamine isothiocyanate (TRITC-6-dUTP): 1 m*M* (Roche Diagnostics).
7. 1X PBS.
8. Bovine serum albumin (BSA; Sigma, St. Louis, MO).
9. *Taq* DNA polymerase (Roche Diagnostics) or Ampli*Taq* (Perkin Elmer, Foster City, CA).
10. 10X *Taq* buffer: 500 m*M* KCl, 100 m*M* Tris-HCl, pH 8.3, 15 m*M* $MgCl_2$.
11. Oligonucleotide primers at 50 pmol/µL.
13. Sterile distilled water.
14. Tween-20 (Roche Diagnostics).
15. Washing buffer (diluted from 20X SSC): 4X SSC, 0.05% Tween-20.
16. 1.5-mL Sterile microcentrifuge tubes (Eppendorf AG, Hamburg, Germany).
17. Cover slips (22 × 32 mm; CML).
18. Copling jar (50 mL).
19. Programmable thermal cycler equipped with a flat pate block (Hybaid Ltd., Teddington, UK).

2.6. Detection and Microscopy

1. DAPI (Sigma).
2. Propidium iodide (Sigma).
3. Antifade solution Vectashield (Vector Labs, Burlingame, CA).
4. Cover slips (20 × 40 mm; CML).
5. Rubber cement.
6. Epifluorescence Microscope Zeiss Axioskop (Carl Zeiss) equipped with ×40 and ×100 Plan Fluo objectives, and with a set of single band-pass filters for DAPI,

FITC, and TRITC single band-pass filter and a triple filter (filter B/G/R, cat. no. 513836) for the simultaneous observation of DAPI, FITC and TRITC signals.
7. Images are captured by a Vysis QUIPS Imaging Analysis System (Vysis, Downers Grove, IL).

3. Methods

3.1. Preparation of Interphase Nuclei Suspension From the Specimens of Fetal and Adult Brain Tissues

1. Place the brain tissue in Petri dish and rinse it in 2 mL of EBBS (*see* **Note 3**).
2. Take a piece of the brain tissue (approx size $3 \times 3 \times 3$ mm) and place it into the homogenizer glass tube. Use the Teflon pestle to homogenize the piece by intense rotating of pestle to produce the liquid-like substance.
3. Add into glass tube containing homogenized tissue 2 mL of PBS containing 0.1% (w/v) of Nonidet P-40 and homogenize for 30 s.
4. Put the substance obtained into a 15-mL plastic (or glass) tube and add 1 mL of 60% (w/v) glacial acetic acid. Leave the mixture obtained for 3 to 5 min at room temperature.
5. Add 9 mL of fixative mixture and centrifuge at 1000*g* for 5 min.
6. Decant supernatant (*see* **Note 4**) and add fixative mixture to 10 mL of total solution volume. Centrifuge at 3000*g* for 8 min.
7. Repeat **step 6** three times (*see* **Note 5**).
8. The obtained suspension can be stored in a 2-mL tube for 6 to 12 mo at –20°C.
9. Put 100 µL of suspension obtained on microscope slide and air-dry for 15 to 20 min.
10. Place slides in dilute pepsin solution (20–100 µL of pepsin) for 3 to 5 min (*see* **Note 6**).
11. Place slides in PBS for 5 min.
12. Dehydrate through series of ethanol (100%, 96%, and 70%, 3 min each) and air-dry. Then proceed to PRINS procedure.

3.2. Preparation of Cell Suspension From Cultured Fetal Neuronal Cells

1. Take 1 to 2 mL of neuronal cells culture (1–2 millions cells per mL) and centrifuge at 3500*g* for 7 min.
2. Wash the precipitation in 1 mL of EBBS by adding it to the tube with precipitant and mixing by inversion for 15 to 30 s. Repeat this procedure using 1 to 1.5 mL of hypotonic solution instead EBBS. Centrifuge at 3500*g* for 7 min.
3. For the decant supernatant, add 1.9 mL of fixative mixture and leave the mixture for 20 min at –4°C.
4. Repeat **step 3** three times. The resulted suspensions should be kept at –20°C.
5. Put 100 µL of suspension obtained on microscope slide and air-dry for 15 to 20 min.
6. Place slides in dilute pepsin solution (20–100 µL of pepsin) for 3 to 5 min (*see* **Note 6**).
7. Place slides in PBS for 5 min.

8. Dehydrate through series of ethanol (100%, 96%, and 70%, 3 min each) and air-dry. Then proceed to chromosomal PRINS labeling procedure.

3.3. Preparation of Formalin-Fixed and Paraffin-Embedded Sections of Autopsy Brain Tissues (see Note 7)

1. The brain tissue section size is supposed to be 7 to 20 μ*M* (*see* **Note 8**). Place the slides with mounted brain tissue sections in 100% xylene at room temperature for 5 min, change xylene and repeat for another 5 min.
2. Rehydrate through series of ethanol (100%, 96%, and 70%, 2 min each) and wash in SSC/detergent mixture for 20 min mixed by inversion periodically (the procedures are made at room temperature).
3. Place the slides into Coplin jar containing 1 *M* NaSCN solution for 3 to 5 h or overnight (*see* **Note 9**).
4. Wash slides by water for a few seconds (without drying).
5. Apply 100 μL of RNase solution in 2X SSC to slide under cover slip and incubate at 37°C for 15 to 30 min (*see* **Note 10**).
6. Place slides in dilute pepsin solution (20–100 μL of pepsin) for 3 to 5 min (*see* **Note 6**).
7. Place slides in 2X PBS, PBS/$MgCl_2$, formaldehyde/PBS/$MgCl_2$, and again in 1X PBS, subsequently each for 5 min.
8. Dehydrate through series of ethanol (100%, 96%, and 70%, 3 min each) and air-dry. Then, immediately proceed to PRINS procedure.

3.4. Quality Control (see Note 11)

1. Drop 5 to 15 μL of prepared suspension on the microscope slide and air-dry.
2. Look into light microscope by using phase contrast option. If the distribution of nuclei is satisfactory (**Fig. 1.1**, **1.5**, **1.6**, **1.10**, **1.12**), skip next two steps.
3. If the distribution of nuclei is too low to analyze (**Fig. 1.2**, **1.3**, **1.8**, **1.9**), centrifuge at 3500*g* for 7 min and decrease the volume in the tube twice. Then mix by inversion and repeat **step 2**. If the distribution of nuclei is satisfactory, skip the next step.
4. If the distribution of nuclei is too dense to analyze (**Fig. 1.4**, **1.7**, **1.11**), centrifuge at 3500*g* for 7 min and decrease the volume in the tube twice. Then, add 0.3 to 0.7 mL of fixative mixture and repeat **step 2**.

Fig. 1. (*opposite page*) Analysis of nuclei density in suspension prepared from adult and fetal brain tissue by phase-contrast microscopy. Suspension of adult brain tissue nuclei, 1.1 to 1.3. (**1**) Dense distribution of nuclei with a high level of superposition. (**2**) Twice-diluted suspension with a optimal nuclei density. (**3**) Suspension diluted three times with a lower nuclei density, 1.4 to 1.6. Suspension of adult brain tissue nuclei. (**4**) Too-dense distribution of nuclei for molecular cytogenetic analysis. (**5,6**) Three times diluted suspension with optimal nuclei density. Suspension of adult brain tissue nuclei, 1.7 to 1.8. (**7**) Extremely dense distribution of nuclei. (**8**) Four times

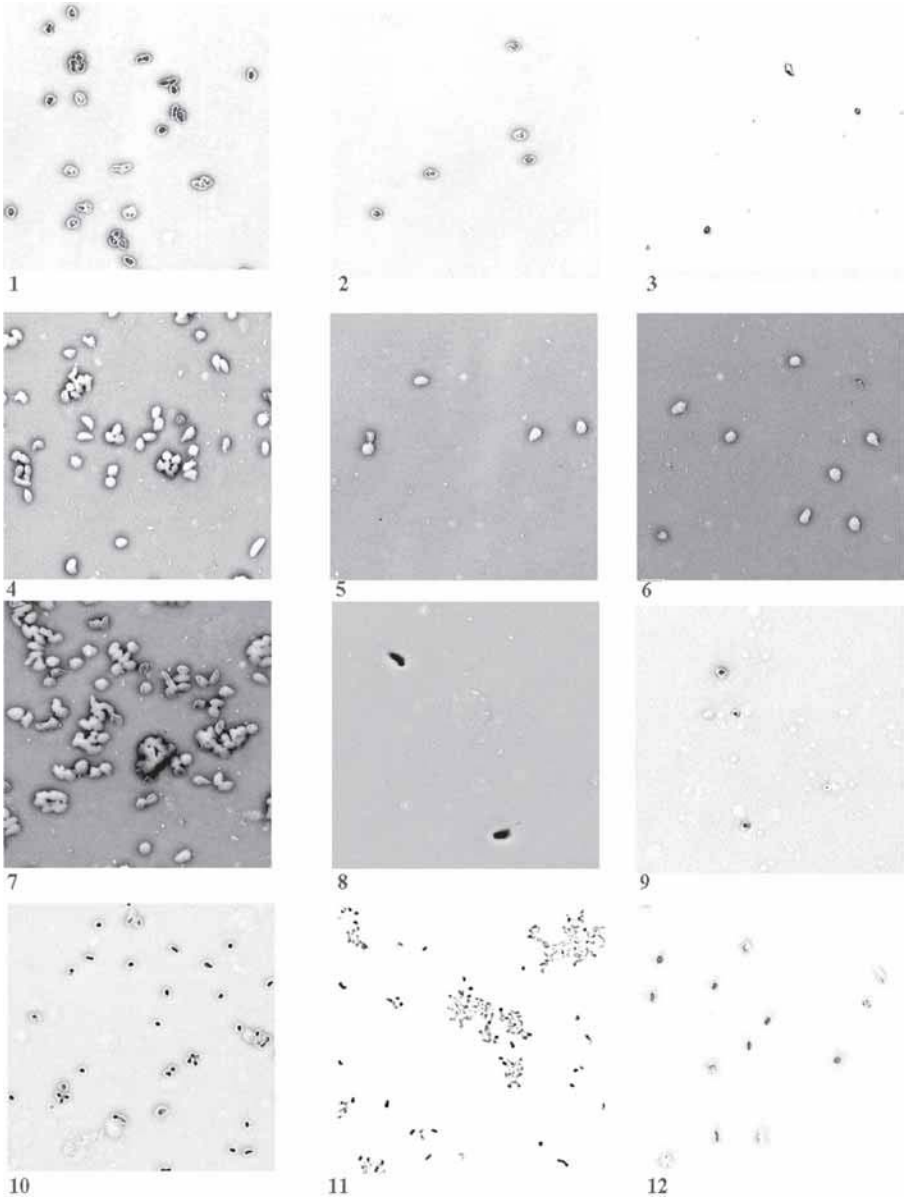

Fig. 1. (*continued from opposite page*) diluted suspension. It should be noted that suspension with low-density nuclei distribution are more suitable for analysis than suspensions with an extremely density of nuclei distribution. Suspension of fetal brain tissue nuclei, 1.9 to 1.10. (**9**) Low-density nuclei distribution. (**10**) Concentrated fetal brain nuclei suspension. Suspension of fetal brain tissue nuclei, 1.11 to 1.12. (**11**) Extremely dense distribution of nuclei. (**12**) Suspension diluted four times.

5. Drop 10 µL of DAPI water solution on microscope slide with prepared suspension of calibrated nuclei density. Put a 24- × 24-mm cover slip on the drop. Examine at the result in a fluorescent microscope.
6. Put the slide into Sudan black solution for 3 min and then rinse in PBS for 3 to 5 min and repeat **step 5** (for postmortem brain suspensions only; *see* **Note 12**).

3.5. PRINS Reaction

In the three-color PRINS procedure, three sequential PRINS reactions are performed, each labeling one specific chromosome. The following labeling order is used: (1) FITC for the first targeted chromosome; (2) TRITC for the second targeted chromosome; and (3) FITC for the third targeted chromosome.

1. Prepare a reaction mixture in a final volume of 50 µL containing: 0.2 m*M* dATP, dCTP, and dGTP, 0.02 m*M* dTTP, 0.02 m*M* FITC-12-dUTP, 50 m*M* KCl, 10 m*M* Tris-HCl, pH 8.3, 1.5 m*M* $MgCl_2$, 0.01% BSA, 200 pmol of oligonucleotide primer; and 2.5 U of *Taq* DNA polymerase. In practice, mix in a sterile microcentrifuge tube: 1 µL of each 1:10 diluted dATP, dCTP and dGTP; 1 µL of the 1:100 diluted dTTP; 1 µL of FITC-12- dUTP, 1 µL of BSA; 5 µL of 10X *Taq* buffer; 0.5 µL of the *Taq* DNA polymerase; 4 µL of the specific primer (for instance, the specific primer for chromosome 1 according to the procedure illustrated in Fig. 1, Chapter 6), and distilled water to 50 µL.
2. Place the reaction mixture under a 22 × 32 cover slip on the denatured slide, and transfer to the heating block of the thermal cycler.
3. Set up the PRINS program and start the reaction. The program consists of a unique 5 min step at the specific annealing temperature of the primer involved for both *in situ* annealing and elongation.
4. While this first reaction is running, prepare the reaction mixture for the second PRINS reaction as described above, but incorporating the specific primer for the second targeted chromosome, and TRITC-6-dUTP.
5. On completion of the program, carefully remove the cover slip from the slide.
6. Wash the slide twice for 2 min at room temperature in 1X PBS.
7. After draining the excess 1X PBS off the slide, and before the slide is completely dry, put the second PRINS reaction mixture on the slide, and cover with a 22 × 32 cover slip.
8. Place the side again on the plate of the thermal cycler.
9. Set up the program for the second PRINS reaction: 5 min at the annealing temperature, specific to the second primer used.
10. Start the program.

No additional denaturation is required after the first PRINS reaction because the chromosomal DNA remains denatured through the PRINS incubations.

11. While this second reaction is running, prepare the reaction mixture for the third PRINS reaction, incorporating the specific primer for the third targeted chromosome and FITC-12-dUTP.

12. At the end of the second reaction, remove the cover slip from the slide and repeat the washing as in **steps 6** and **7**.
13. Before the slide is completely dry, put the third PRINS reaction mixture on the slide, and cover with a 22 × 32 cover slip.
14. Place the slide on the thermal cycler.
15. Set up the program for the third PRINS reaction: 5 min at the annealing temperature, specific to the third primer used.
16. Start the program.
17. At the end of this third reaction, the slide is transferred to 4X SSC, 0.05% Tween-20 for two washes (3 min each) at room temperature with gentle agitation.
18. Drain the excess washing solution off the slide.
19. Mount the slide in Vectashield antifade solution containing either DAPI (0.3 µL/mL) or a mix of propidium iodide (0.3 µL/mL) and DAPI (0.3 µL/mL).
20. Cover with a 22 × 40 cover slip and seal the cover slip with rubber cement.
21. Examine the slide under the epifluorescence microscope, preferentially using first the triple band-pass filter, and confirming the coloration of the fluorescent spot with single band-pass filters.
22. The slide may be stored in the dark at 4°C for several months.

4. Notes

1. Although some protocols propose the use of ethanol instead of methanol, because of the extreme toxicity of this compound, the fixative mixture prepared using methanol is optimal for tissue preparation (especially brain tissues) for molecular cytogenetic analysis.
2. Instead of Tween-20, another mild detergent, such as Triton-X100, may be used (0.1% of Triton-X 100 should be added in SSC solution).
3. This step can be skipped when postmortem brain samples are processed.
4. It is recommended that one leave approx 2 mL of liquid above the pseudo-solid phase.
5. The number of repetitions of **step 6** should be assessed empirically and principally depends on the age of sample (e.g., freshly prepared autopsy requires to repeat fixation three times; however, for autopsies older than 48 h, repeating the fixation step five or six times is recommended).
6. The conditions of pepsin treatment generally are selected empirically; however, it should be noted that some of formalin-fixed and paraffin-embedded sections of brain could require a long-term pepsin treatment (as long as 7 min).
7. The protocol proposed also can be applied to frozen sections of brain tissues. For frozen sections, skip **steps 1–4**.
8. Tissue section should be 7 to 14 µ*M* to provide a sufficient amount of nuclei to analyze and reduce the problem of damaged nuclei because the size of brain tissue cells is quite large compared with a number of another somatic tissue cells.
9. The leading cause of problems in preparation of formalin-fixed autopsic brain tissue is the incomplete DNA–peptides complex disruption during processing of the slides for molecular-cytogenetic analysis. The treatment with NaSCN solution should be conducted and conditions of the step are selected empirically.

10. RNase treatment is especially recommended for frozen sections of brain tissue. The step can be skipped for frozen sections kept in liquid nitrogen or fresh paraffinized sections as well as when DNA probes for nontranscribed DNA sequences are applied *(6)*.
11. Quality control procedure can be applied to cell suspensions only.
12. Sudan black solution is used for postmortem brain samples only. Postmortem brain suspensions usually are characterized by increased level of lipofuscin-like autofluorescence. Treatment with Sudan black solution reduces considerably the lipofuscin-like autofluorescence and does not affect specific fluorescence labels *(7)*.

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13

PRINS on Plant Chromosomes

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Summary

The introduction of primed *in situ* labeling (PRINS) into plant cytogenetics provided a novel means for fast and highly specific visualization of DNA sequences on chromosomes and in interphase nuclei. Although the technique does not reach the sensitivity of fluorescence *in situ* hybridization that is needed for detection of single-copy targets, it is superior in its speed and simplicity. Thus, the main applications of PRINS include fluorescent labeling of repeated DNA sequences, such as ribosomal DNA and satellite repeats, which are used to discriminate individual chromosome types within karyotypes and to assess the purity of chromosome fractions separated by flow-sorting. Recently, an application of PRINS for the discrimination of sequence subfamilies of satellite repeats has been developed that takes advantage of the sensitivity of *Taq* polymerase to mismatches between 3'-end of the primer and the template sequences. This approach allows the distinguishing of sequences that differ in only a few nucleotides and has proved to be valuable for studies of satellite DNA evolution in plants.

Key Words: Plant chromosomes; repetitive sequences; satellite DNA; flow cytometric sorting.

1. Introduction

Primed *in situ* labeling (PRINS) was first applied to examine the molecular structure of human and animal chromosomes where it proved to be a useful alternative to established fluorescence *in situ* hybridization protocols ***(1,2)***. Initial efforts to apply this technique to plants were stimulated by a need for a simple procedure to fluorescently label specific DNA repeats on mitotic chromosomes in liquid suspension before their analysis using flow cytometry ***(3,4)***. Subsequent studies aimed at improving the sensitivity and specificity of the procedure ***(5)***, including a modification of PRINS called cycling PRINS ***(6,7)***. Despite these significant advances, the sensitivity of PRINS on plant chromosome preparations remains lower when compared with fluorescence *in situ*

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hybridization, and the method does not allow for the reliable visualization of short single- and low-copy targets ***(8)***.

The most productive applications of PRINS have been the investigation of the genomic distribution of plant satellite repeats ***(9–11)*** and the analysis of flow-sorted chromosomes ***(12)***. Because of its speed, PRINS has been the method of choice for identifying flow-sorted chromosomes and for determining the purity of sorted chromosome fractions in a number of species, including garden pea ***(13)***, barley ***(14)***, and wheat ***(15,16)***. In addition, PRINS was found useful in the development of molecular karyotypes, in which labeled satellite repeats can be used as cytogenetic landmarks and allow the identification of morphologically similar chromosomes ***(17)***. Recently, it has been demonstrated that PRINS can discriminate and, thus, specifically label, closely related repeat subfamilies ***(11)***. This labeling was achieved by designing oligonucleotide primers for the reaction such that their 3'-ends corresponded to sequences specific for the individual subfamilies. Because the sequence similarity between the primer 3'-end and the target is crucial for its extension by *Taq* polymerase, any mismatch in this region greatly reduces its performance.

In this chapter, we provide a protocol for PRINS labeling of satellite repeats on plant chromosomes. As an example, we demonstrate labeling of VicTR-B repeats ***(10)*** on chromosomes of a legume plant *Vicia grandiflora*. This species genome contains several subfamilies of the VicTR-B sequences differing in short (2–4 bp) variable regions (J. Macas et al., 2005, unpublished observations). Targeting the primer to one such region allows the visualization of the chromosome distribution of that specific subfamily, whereas using a probe derived from conserved sequence domains labels all loci containing the VicTR-B repeats. The protocol involves denaturation of chromosomal DNA directly in the PRINS reaction mixture, annealing of unlabeled oligonucleotide primers to corresponding chromosomal targets, and their extension by *Taq* polymerase in the presence of fluorescently labeled dNTPs. Only a single cycle of DNA synthesis is required to produce sufficient signals, which makes the labeling procedure very short (approx 1 h).

2. Materials

2.1. Chromosome Preparation

1. Vigorously growing roots of *Vicia grandiflora*.
2. Digestion mix: 2.5% pectolyase Y-23 (MP Biomedicals) and 2.5% cellulase "Onozuka" R-10 (Yakult Pharmaceutical Industry, Tokyo, Japan) dissolved in 75 m*M* KCl and 7.5 m*M* EDTA, pH 4.0. Store the mix in 200-µL aliquots at –20°C.
3. 75 m*M* KCl.
4. Microscope slides and cover slips (Menzel GmbH., Braunschweig, Germany).
5. Acid-cleaned slides: Working in a fume hood, prepare 100 mL of saturated solution of $K_2Cr_2O_7$ in deionized water and slowly add 50 mL of concentrated sulfu-

ric acid with constant stirring. Submerse the glass slides individually into the solution and leave for at least 1 h, then wash the slides in several changes of excess water until the chromic acid is removed (the solution is no longer golden-brown). Submerse the slides into 65% HNO_3 for 30 min and wash them in deionized water. Store the slides in 96% ethanol at room temperature and air-dry before use.

6. Acetic acid, glacial (ICN).
7. Ethanol, 70% and 96%.
8. Liquid nitrogen.

2.2. PRINS Labeling

1. Frame-Seal incubation chambers, 15 × 15 mm, 65-µL capacity (MJ Research).
2. *Taq* DNA Polymerase, 5 U/µL (Promega).
3. 10 X Polymerase chain reaction (PCR) buffer: 500 m*M* KCl, 100 m*M* Tris-HCl, pH 9.0, 1% Triton X-100 (Promega).
4. 25 m*M* $MgCl_2$ (Promega).
5. Dideoxy-nucleotide mix: 0.1 m*M* of each of ddATP, ddCTP, dd-GTP, and ddTTP (all Boehringer Mannheim).
6. Washing solution: 1X PCR buffer, 4 m*M* $MgCl_2$.
7. Labeling mix: 1.0 m*M* of each of dATP, dCTP, dGTP; 0.17 m*M* dTTP (Promega); and 0.08 m*M* Alexa Fluor 488-5-dUTP (Molecular Probes).
8. Oligonucleotide primers: VicTR-B_cons-F (5'-ATA TAA GTC TTC ARA AAA T-3'), VicTR-B_cons-R (5'-GAA GAC TTA TAT TCA CTT-3'), and VicTR-B_VG-3R (5'-TTC ACC ATA TTT TCT CAW GAT TTA TGC C-3') diluted to 4 µ*M* in sterile deionized water.
9. 2X Standard saline citrate (SSC): 0.3 *M* NaCl, 0.03 *M* sodium citrate, pH 7.0 (sterile).

2.3. Counterstaining and Microscopy

1. McIlvaine's buffer: 164 m*M* Na_2HPO_4, 18 m*M* citric acid, pH 7.0.
2. DAPI stock solution: dissolve DAPI (Sigma) in deionized water to concentration of 100 µg/mL. Filter through a 0.22-µm filter (Millipore) and store in aliquots at –20ºC.
3. 4X SSC/Tween: 0.6 *M* NaCl, 0.06 *M* sodium citrate, pH 7.0, 0.2% (v/v) Tween-20 (Sigma).
4. Antifade: Vectashield mounting medium (Vector Laboratories).
5. Parafilm.

3. Methods

3.1. Chromosome Preparation

1. Excise root tips (10–15 mm long) and pretreat them in 15 µ*M* oryzalin for 2–4 h at 25°C in the dark.
2. Remove the pre-treatment solution and fix the root tips in ethanol:acetic acid (3:1) for 2 h at 4°C and store in 70% ethanol at –20°C for up to 18 h (*see* **Note 1**).

3. Wash 100 root tips fixed in ethanol:acetic acid for at least 30 min in 500 mL of deionized water to remove the fixative.
4. Using a scalpel, remove as much of the nonmeristematic tissue as possible (i.e., remove the root cap and use only the terminal part of the root tip).
5. Transfer the meristems into 200 µL of enzyme solution and digest their cell walls at 27°C until the material is soft but still maintains its morphology (*see* **Note 2**).
6. Wash the meristems in 10 mL of 75 m*M* KCl for at least 15 min.
7. Transfer the material into 12 µL of 45% aqueous acetic acid on acid-cleaned slide (*see* **Note 3**); use three root tips for each slide.
8. Gently disperse the tissue, apply a clean 24- × 24-mm cover slip (*see* **Note 4**) and squash the protoplasts using a pressure adequate to restrict blood to the thumb nail.
9. Immerse the preparation into liquid nitrogen, then flick off the cover slip with a scalpel or razor blade.
10. Dehydrate the preparation in an ethanol series (2 × 5 min in 70% and 2 × 5 min in 96% ethanol, respectively) and allow the slide to air-dry.
11. Store the slides in a dessicator at –20°C (*see* **Note 5**).

3.2. PRINS Labeling

1. Attach the Frame-Seal chamber to the slide region containing chromosomes and incubate the slide for 5 min at 60°C to improve frame adhesion.
2. For each slide, prepare 70 µL of PRINS mix consisting of 40.2 µL of H_2O, 7 µL of 10X PCR buffer, 7 µL of labeling mix, 11.2 µL of 25 m*M* $MgCl_2$, 3.5 µL of 4 µ*M* primer (*see* **Notes 6** and **7**), and 1.1 µL of *Taq* polymerase. Keep the mix on ice during preparation and until application to the slide.
3. Dideoxy-blocking of 3'-ends (*see* **Note 8**): For each slide, prepare 100 µL of blocking mix consisting of 63 µL of H_2O, 10 µL of 10X PCR buffer, 10 µL of dideoxynucleotide mix, 16 µL of 25 m*M* $MgCl_2$, and 1 µL of *Taq* polymerase. Pipette the mix into the frame and cover it with a 24- × 24-mm cover slip (do not remove the top liner from the frame).
4. Incubate for 15 min at 72°C on a heating block using a humidity chamber (*see* **Note 9**). Equilibrate the slide to room temperature and remove the blocking mix using a pipet.
5. Apply 100 µL of washing solution and incubate at room temperature for 3 min. Remove the mix and repeat the washing step two more times (*see* **Note 10**). Upon completion, remove the washing solution as completely as possible.
6 Remove the top liner from the Frame-Seal chamber, put the slide on a Petri dish chilled on ice, and pipet 70 µL of the PRINS mix at one end of the chamber. Seal the frame using polyester cover and transfer the slide onto a heating block (*see* **Note 11**) preheated to 94°C.
7. Immediately run the incubation profile consisting of DNA denaturation for 3 min at 94°C, primer annealing for 5 min at 45°C (*see* **Note 12**), heating to 72°C at the rate of 0.1°C/s, and primer extension for 30 min at 72°C.
8. Remove the frame and wash the slide in 2X SSC for 3 min at room temperature.

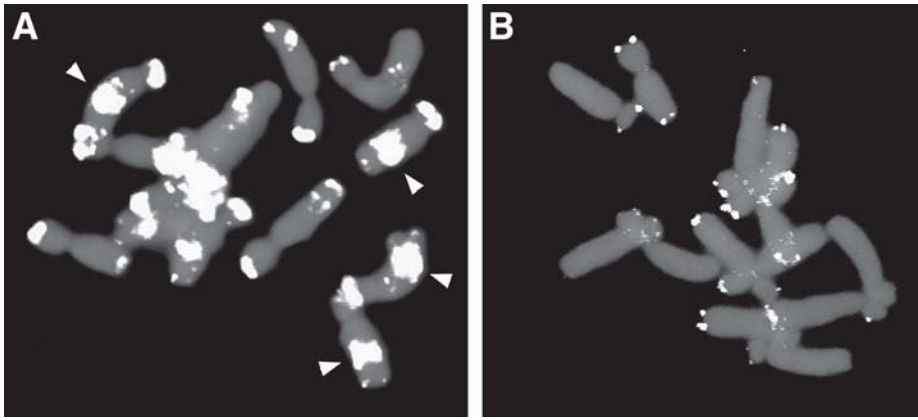

Fig. 1. PRINS labeling of VicTR-B repeats on *Vicia grandiflora* chromosomes. The reaction was performed by using primers VicTR-B_cons-F and VicTR-B_cons-R, allowing labeling of all VicTR-B sequences (**A**) or by using a single primer, VicTR-B_VG-3R, designed to visualize only a subfamily of the repeats (**B**). Note that this repeat subfamily is predominantly located in (sub)telomeric chromosome regions and is absent in other regions containing VicTR-B sequences (e.g., the prominent intercalary bands marked with arrowheads on A are not labeled on B). The chromosomes were counterstained with DAPI and appear gray, the PRINS signals are white.

3.3. Counterstaining and Microscopy

1. Dilute DAPI stock solution to 2 μg/mL in McIlvaine's buffer.
2. Add 80 μL of DAPI working solution to each slide, cover with a piece of Parafilm and incubate for 10 min at room temperature.
3. Remove Parafilm and wash slides for 5 to 10 s in 80 mL of 4X SSC/Tween in a Coplin jar.
4. Mount the preparation using a drop of antifade solution.
5. Observe the chromosomes using an epifluorescence microscope equipped with appropriate filter sets (e.g., UV-2A for DAPI and B-2A for Alexa Fluor 488) on Nikon Eclipse-600 microscope. Typical results are shown in **Fig. 1**.

4. Notes

1. It is very useful to synchronize cell divisions within the meristems before their fixation and chromosome preparation. Cell cycle synchronization protocols are available for a wide range of species ***(18)***.
2. The digestion takes 20 to 30 min at 27°C for *Vicia* meristems. However, appropriate digestion time may be different for other species and has to be determined empirically.
3. If the chromosomes and nuclei do not adhere well to the slides and are being lost during subsequent treatments, use slides coated with reagents that improve chromosome adhesion to the surface (e.g., Super Frost Plus slides, Menzel Gmbh.).

4. Do not use acid-cleaned cover slips to which chromosomes would stick.
5. The preparations can be used the next day or stored for up to several months.
6. There is only one primer used in this reaction; however, two or more primers can be used simultaneously to provide better coverage of the target sequence. In that case, the same amount of each primer (3.5 µL) is used and the volume of H_2O in the mix has to be adjusted accordingly.
7. It is strongly recommended to also run a control reaction using the PRINS mix without primers. This reaction will allow clear discrimination between primer-induced and eventual unspecific background signals.
8. Pretreatment of slides with dideoxynucleotides reduces unspecific background by blocking free 3'-ends of chromosomal DNA generated during chromosome preparation. After the incorporation of ddNTPs, these ends can no longer be extended in the presence of labeled nucleotides in the subsequent PRINS reaction. Because this treatment improves signal-to-background ratio, it is especially useful for the detection of less-abundant targets that could be obscured by the background. However, in most cases, it is not necessary and **steps 3** to **5** may be omitted.
9. A humidity chamber can be made by adding a piece of water-soaked tissue to the heating block equilibrated to 72°C approx 10 to 15 min before the slide incubation (it is necessary to use the block mounted within a closed chamber, *see* **Note 11**). This is required to avoid evaporation of the blocking mix as the cover slip is not tightly attached to the Frame-Seal chamber.
10. This treatment removes unincorporated ddNTPs that would otherwise block the PRINS reaction.
11. There are a variety of instruments equipped with flat block chambers suitable for slide incubation; in our laboratory, we use MJ Research PTC-200 with Twin Tower module (MJ Research).
12. Annealing temperature depends on the primer length and base composition and is usually between 45 and 65°C. The primers described in this chapter were annealed at 45°C (VicTR-B_cons-F, VicTR-B_cons-R) and 65°C (VicTR-B_VG-3R), respectively.

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14

Dual-Color PRINS for *In Situ* Detection of Fetal Cells in Maternal Blood

Kada Krabchi, Macoura Gadji, Ju Yan, and Régen Drouin

Summary

Fetal nucleated cells circulating in the peripheral blood during pregnancy are potential targets for noninvasive genetic testing. Fluorescence *in situ* hybridization (FISH) frequently is used to quantify the total number of fetal cells in peripheral blood of pregnant women. We describe an alternative molecular cytogenetic procedure that is the primed *in situ* labeling (PRINS). This technique consists of annealing oligonucleotides specific to individual chromosome targets and *in situ* elongation using *Taq* DNA polymerase to incorporate labeled dUTPs. The sites of the newly synthesized DNA sequences were revealed as fluorescent signals using an immunochemical reaction. The dual-color PRINS was specifically performed for simultaneous detection of two chromosome targets, X and Y. The fluorescent signals corresponding to chromosomes X and Y were displayed as red and green color spots, respectively. The sensitivity and specificity of PRINS are similar to FISH and allow us to efficiently and reliably detect fetal cells in maternal blood. Moreover, dual-color PRINS is faster and more cost-effective than FISH.

Key Words: Primed *in situ* labeling; multi-PRINS; fetal cells; molecular cytogenetics; human chromosomes.

1. Introduction

Primed *in situ* labeling (PRINS) originally was designed by Koch et al. in 1986 for the investigation of minute sequence structures within primate α-satellite DNA ***(1–3)***. Since that time, numerous technical variants have been described, and a wide variety of targets have been detected ***(4,5)***. PRINS reaction is defined as a nonisotopic *in situ* labeling method for the rapid and efficient detection of interphase and metaphase DNA. This method is based on the annealing of specific oligonucleotide primers, derived from chromosome-specific subsets of DNA sequences, to the denatured DNA, followed by subsequent primer extension using *Taq* DNA polymerase. Specific labeling was

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obtained in fetal nucleated cells prepared from a sample of maternal peripheral whole blood. Unlike the conventional fluorescence *in situ* hybridization (FISH) technique, PRINS reaction is a sensitive molecular cytogenetic technique that has empowered the conclusive detection of the presence of the few and rare fetal nucleated cells circulating in maternal blood. We tested the dual-color PRINS reactions to detect sexual chromosomes of fetal cells according to Yan et al. multi-PRINS protocol ***(6)*** on blood samples obtained from pregnant women at the second trimester of gestation. The purpose of this study was to determine the exact number of fetal nucleated cells per unit volume of maternal blood. Using specific primers for chromosomes X and Y, we proceeded to the primer–nucleotide–hapten mix annealing stage, followed by specific antibody–fluorophore complex detection. In our previous study, using two simple and efficient methods (FISH and PRINS), we concluded that the exact number of fetal nucleated cells per unit volume of maternal blood (1 mL), fluctuated between 2 and 6 during the second trimester of pregnancy ***(7)***. These results suggest that PRINS might be a powerful tool that is more specific, considerably faster, and less expensive than classical FISH techniques. Our experiments show that the PRINS reaction is potentially of great interest as an alternative to FISH for nucleated fetal cell analysis. Furthermore, PRINS could be useful in noninvasive prenatal testing.

2. Materials

2.1. Preparation of Fetal Cells

1. Maternal whole blood samples were obtained by antecubital venepuncture in a *VACUTAINER*® Sodium Heparin tube (Becton Dickinson *VACUTAINER* Systems, Franklin Lakes, NJ).
2. *VACUTAINER* K_2 EDTA 3.6-mg tube (2 mL; Becton Dickinson *VACUTAINER* Systems).
3. Sodium chloride injection UPS 0.9% solution (10 mL; Abbot Laboratories, Limited, Montreal, Canada).
4. Falcon polypropylene plain tubes (15 mL).
5. Hank's balanced salt solution (HBSS, MULTICELL, 1X without Ca, Mg, and Phenol Red, WISENT Inc.).
6. Centrifuge (ICE centrifuge, HN-SII model A Division of Damon, USA).
7. Hypotonic solution: 0.075 *M* KCl (0.56 g/L).
8. Carnoy fixation solution: 3 v methanol:1 v glacial acetic acid.
9. Thermotron environmental control unit (CDS-5, Thermotron, Amsterdam, Holland ***[8]***).
10. Methacarn solution:methanol:chloroform:glacial acetic acid (6:3:1 v/v).

2.2. PRINS Reaction

2.2.1. Equipment

1. Glass slides (25 × 75 × 1 mm; VWR Scientific, Inc.) and selected micro cover glasses, cover slips (24 × 30 mm) (VWR Scientific, Inc.). The slides must be cleaned by soaking in series of ethanol solutions followed by polishing with a clean piece of muslin and wiped dry using lint-free paper before dropping the cells on the slides.
2. Variable volume micropipets ranging from 0 µL to 1000 µL.
3. Microcentrifuge tubes (1.5 mL).
4. Plastic and glass Coplin jars, or other suitable containers for washing the slides.
5. 50-mL Centrifuge tube.
6. 15-mL Polypropylene conical tube 17 × 120-mm style (Becton Dickinson).
7. Forceps.
8. Humidified slide chamber.
9. Water bath with accurate temperature control at 37°C.
10. Centrifuge (ICE centrifuge).
11. Thermotron environmental control unit (CDS-5, Thermotron)
12. Incubator at 37°C.
13. Light microscope.
14. Thermal cycling machine equipped with a flat block thermal cycler PTC (Program Temperature Control).

2.2.2. Solutions

1. 20X Standard saline citrate (SSC): 3.0 *M* NaCl, 0.30 *M* tri-sodium citrate, pH 7.2–7.4.
2. 2X SSC, pH 7.2–7.4 (diluted from stock 20X SSC): Store these solutions at ambient temperature and discard after 6 mo or sooner if the solution appears cloudy or contaminated.
3. 70%, 80%, and 100% ethanol.
4. 10X Phosphate-buffered saline (PBS): 1360 m*M* NaCl, 20 m*M* KCl, 106 m*M* Na_2HPO_4, 15 m*M* KH_2PO_4, pH 7.2–7.4.
5. 1X PBS, pH 7.2–7.4 (diluted from stock 10X PBS): Store PBS solutions at ambient temperature and discard after 6 mo or sooner if the solution appears cloudy or contaminated.
6. Tris-EDTA (TE) buffer: 10 m*M* Tris-HCl, pH 8.0; 1 m*M* EDTA.
7. Denaturation solution: 70% formamide/2X SSC. Prepare denaturation solution by mixing thoroughly 35 mL of deionized formamide (ultrapure grade, Fluka), 5 mL of 20X SSC, and 10 mL of distilled water in a glass Coplin jar, pH 7.2–7.4. Between usage, store denaturation solution at 2–8°C and discard after 3 to 5 d.
8. Washing buffer: 4X SSC (diluted from stock 20X SSC), 0.05% Triton X-100 or 0.2% Tween-20.
9. Blocking buffer: 5% dried skimmed milk powder in wash buffer. Centrifuge for 2 min and use the supernatant, which can be stored at 4°C for up to 3 d.

10. PRINS reaction solution: For each slide, in a 1.5-mL microtube, mix: 5 µL of 10X polymerase chain reaction buffer (Roche Molecular Biochemicals), dATP, dCTP), d-GTP, and dTTP working solution (4 µL of each), primer solution (2 µL), 1 m*M* biotin-dUTP (1 µL) or 1 m*M* digoxigenin-dUTP (1 µL), 2.5 µL of glycerol (*see* **Note 1**), 23 µL of distilled water, and 0.5 µL of *Taq* DNA polymerase (Roche Molecular Biochemicals; add immediately before starting the PRINS reaction).

2.2.3. Primers (see **Table 1**)

1. Resuspend the oligonucleotides in TE buffer for a concentration of 100 µ*M* as a primer stock solution (*see* **Note 2**).
2. Mix 5 µL of primer stock solution with 95 µL of TE buffer or water for a 5 µ*M* primer working solution.

2.2.4. dNTPs

1. dCTP, dGTP, and dATP working solution: Dilute 100 m*M* stock solution of each of dNTP (Roche Molecular Biochemicals) to a concentration of 2.5 m*M* by mixing 1 µL of each dNTP with 39 µL of sterile distilled water.
2. dTTP working solution: Dilute 100 m*M* stock solution dTTP (Roche Molecular Biochemicals) to a concentration of 0.25 m*M* by mixing 1 µL of dTTP with 399 µL of sterile distilled water.
3. 1 m*M* of biotin-16-dUTP or biotin-11-dUTP (Enzo), and digoxigenin-11-dUTP (Roche Molecular Biochemicals).

2.3. Detection

1. 1% Avidin-fluorescein DCS (Vector Laboratories, Burlingame, CA) and 1% anti-dig-rhodamine (Roche Molecular Biochemicals; diluted with blocking buffer).
2. 1% Avidin-rhodamine (Roche Molecular Biochemicals) and 1% antidigoxigenin-fluorescein (Roche Molecular Biochemicals; diluted with blocking buffer). Either of the two aforementioned detection mixes can be used according to the selected labeling protocol for chromosome targets detection (*see* **Note 3**).
3. Counterstaining: In 1 mL of 0.1 *M* Tris-HCl, pH 7, dissolve 125 ng of DAPI; (Sigma), 1 mg of *p*-phenylenediamine, and then mix with 9 mL of glycerol.
4. Fluorescence microscope equipped with appropriate and optimal filter sets (DAPI/Green dual bandpass, DAPI/Orange dual bandpass) and connected to an image system (e.g., ISIS 2 in MetaSystems, Belmont, MA).

3. Methods

3.1. Preparation of Fetal Cells

1. Before amniocentesis, the maternal blood samples were obtained by antecubital venepuncture in a *VACUTAINER* sodium heparin tube (Becton Dickinson).
2. Add 3 mL of sodium chloride, 0.9% solution, into a *VACUTAINER* K_2 EDTA 3.6-mg lavender-top tube.

Table 1
Sequences of Oligonucleotide Primers Used in Dual-PRINS Technique

Name	Location	Sequence	Annealing temperature	Ref.
Xc	X	GTTCAGCTCTGTGAGTGAAA	65°C	***9***
D599 (27-mer)	DYZ1	TGGGCTGGAATGGAAAGGAATCGAAAC	56°C	***10***
D600 (27-mer)	DYZ1 (pair for D599)	TCCATTCGATTCCATTTTTTTCGAGAA	56°C	***10***

3. Immediately after blood sampling (*see* **Note 4**), mix 3 mL of whole blood with 3 mL of sodium chloride, 0.9% solution, taken from *VACUTAINER* K_2 EDTA tube and quickly aliquote six equal 1-mL volumes of into 6 Falcon polypropylene plain tubes (15 mL).
4. To wash the cells, 11 mL of Hank's Balanced Salt Solution (HBSS) is added to each 15-mL tube. This step is repeated twice.
5. After mixing, the cells are centrifuged at 500*g* for 7 min.
6. The platelet-rich supernatant is discarded, and the cells are resuspended in a hypotonic solution (0.075 *M* KCl) and incubated at 37°C for 10 to 15 min.
7. The cells are then centrifuged for 5 min at 667*g*.
8. Two Carnoy's fixations are performed using the standard methodology. The pellet is resuspended in 1 mL of fresh Carnoy.
9. Cell suspensions may be pooled in three microtubes (the volume equivalence of blood per microtube is 1 mL) and stored in Carnoy at –20°C for several months.
10. Spread 15 µL of fixed nucleus suspension onto cleaned slides in a modified Thermotron environmental control unit at an optimal temperature (25°C) and humidity (36% ***[8]***). After that, immerse and agitate the slides in methacarn solution for 20 to 30 s (*see* **Note 5**). Then, put the slides on racks and keep them within Thermotron chamber until they dried. They can be used immediately or stored at –20°C for several weeks (*see* **Note 6**).
11. Denature slides in 70% formamide/2X SSC at 70°C for 2 min and successively pass them through 70%, 80%, and 100% ethanol for 2 min each at –20°C. After air-drying, the slides are ready for the PRINS procedure.

3.2. PRINS Reaction

1. Start the PRINS by adding the first reaction solution (50 µL) containing a specific X-chromosome primer and one of the labels onto a slide (*see* **Note 3**). Cover the slide with a cover slip and place the slide onto the flat block of a thermocycler.
2. Perform the annealing and extension steps according to the different primers used for the detection of different chromosome targets (*see* **Note 2**).
3. Wash the slide briefly in 1X PBS solution for 2 min after the first PRINS reaction ending.
4. Add the second reaction solution containing the Y specific primers for the chromosome Y and an alternately labeled dUTP on the same slide (*see* **Note 7**).
5. Perform the second PRINS reaction as in **step 2** (*see* **Note 8**).
6. Wash the slide in wash buffer for 5 min with gentle agitation.

3.3. Detection

1. Mount the slide with 100 µL of blocking buffer and a cover slip. Leave the slide at room temperature for 5 min.
2. Remove the cover slip, drain blocking buffer from the slide, and apply 100 µL of a mix of detection solution to the slide.
3. Apply 100 µL of a detection solution mix (1% avidin-fluorescein and 1% antidig-rhodamine diluted with blocking buffer or 1% Avidin-rhodamine and 1%

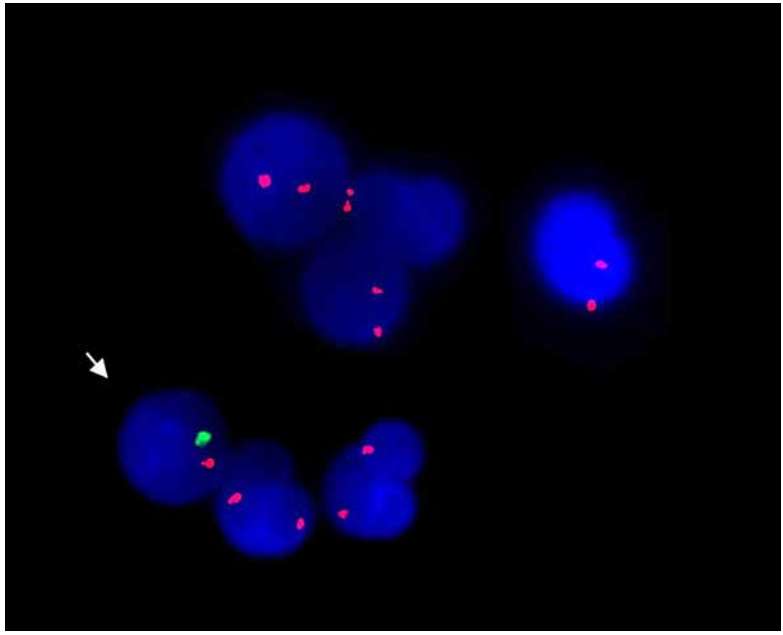

Fig. 1. Dual-color PRINS labeling on chromosome-X (red signal) and Y (green signal) of a fetal nucleus from maternal blood from a woman carrying a male fetus. The white arrow indicates a male fetal cell surrounded by female maternal nuclei. (Please *see* color insert following p. 48.)

Antidigoxigenin-fluorescein diluted with blocking buffer). Either of the two aforementioned detection mixes can be used according to the selected labeling protocol for the detection of the two chromosome targets.

4. Place on a new cover slip and incubate the slide in a moist chamber at 37°C for 30 min.
5. Remove cover slips gently by tilting the glass slide, and let the cover slip slide off.
6. Wash the slide three times in wash buffer at room temperature for 5 min each with gentle agitation. Like that, excess antibody is removed (*see* **Note 9**).
7. After the slide air-drying in dark, apply 10 to 20 µL of DAPI counterstaining solution to the slide and cover the slide with a cover slip.
8. Male fetal cells were identified by the presence of two fluorescent signals of different colors (**Fig. 1**). The fetal and maternal cells showing representative signals were recorded using a Compulog IMAC-CCD S30 video camera module and analyzed using the *in situ* imaging system mounted on the fluorescence microscope.
9. Given the brightness of the signals, the slides are scanned at a magnification of ×400, but every cell showing two signals of different colors are examined at ×1000 magnification.

4. Notes

1. The order in which primers are used is important. We recommend using the X chromosome primer in the first reaction and the Y primers in the second reaction. Therefore, if the first labeling uses biotin-dUTP and second labeling uses digoxigenin-dUTP (bio-dig) for X and Y chromosome targets, respectively, an appropriate fluorochrome mix should be avidin–rhodamine/antidigoxigenin–fluorescein. Conversely, if the labeling order is dig-bio, the fluorochrome mix should be antidigoxigenin–rhodamine/avidin–fluorescein. The principle of selecting a relatively weak fluorochrome for the last PRINS target detection is critical for double-PRINS.
2. The lyophilized oligonucleotide is stable at –20°C for 1 yr or longer. It is generally accepted that oligonucleotides dissolved in TE are stable for at least 6 mo at –20°C or 4°C. Oligonucleotides dissolved in water are stable for at least 6 mo at –20°C. Do not store oligonucleotides in water at 4°C. TE is recommended when compared to deionized water because the pH of the water is often slightly acidic and can cause hydrolysis of the oligonucleotides.
3. For X chromosome detection, the PRINS program should be annealing at 65°C for 10 min, with an extension at 72°C for 10 min. For Y chromosome annealing, 56°C for 10 min and an extension to 72°C for 10 min is advised ***(4)***.
4. Blood specimens were processed at the latest, within 2 h of venepuncture. This is required to avoid fetal cell apoptosis as the result of their supposed fragility in maternal blood.
5. The efficiency of the PRINS procedure relies highly on the temperature and humidity conditions when preparing slides. The optimal conditions for dropping cells onto slides can be reached by using a Thermotron (CDS-5 ***[8]***) or in a temperature/humidity-adjustable chamber. The optimal conditions may vary from laboratory to laboratory and should be determined by pretesting. Ideally, the nuclei on the slide should show a gray color, and no reflective nuclei or bright rings around any nuclei should be observed. High slide background noise could be caused by cellular debris generated during sample preparation. Therefore, you must wash the cell pellet with fresh fixative twice and repeat the slide dropping procedure. In addition, treat the slides with methacarn to make the DNA sample more accessible to the primers by the removal of loosely bound protein and discarded cytoplasmic debris.
6. The use of fresh prepared slides is recommended. Old slides may lead to reduced sensitivity and greater variability. Similarily, longer storage can give rise to background signals.
7. Use double primers for the same locus of chromosome Y to increase the efficiency of PRINS reaction.
8. No denaturation is required after the first PRINS reaction because the nucleic DNA remains denatured throughout the PRINS incubation.
9. Stringent washing in SSC obtains minimal background noise.

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15

PRINS as an Efficient Tool for Aneuploidy Assessment in Human Oocytes and Preimplantation Embryos

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Summary

An ultrarapid three- and four-color primed *in situ* (PRINS) procedure has been developed for rapid chromosome identification and aneuploidy assessment on isolated cells. Based on the direct *in situ* mixing of fluorochromes (fluorescein isothiocyanate, tetramethylrhodamine isothiocyanate, Cascade Blue), this multicolor PRINS procedure is described on unfertilized human oocytes and isolated human blastomeres.

Key Words: PRINS; aneuploidy; oocyte; blastomere.

1. Introduction

Among all the adaptations of molecular cytogenetic procedures, the *in situ* chromosomal analysis of isolated human oocytes and blastomeres constitutes an important challenge because schromosomal abnormalities are known to be the major reason for before and after implantation human embryo wastage, with a significant prevalence of female meiotic segregation errors. To date, fluorescence *in situ* hybridization and primed *in situ* labeling (PRINS) have been successfully adapted on isolated human gametes ***(1–3)***. Because of its relative simplicity and the commercial availability of numerous DNA probes, fluorescence *in situ* hybridization has become the standard technique for *in situ* chromosomal investigations. However, the PRINS reaction offers a fast alternative approach based on the use of short, unlabeled, and chromosome-specific primers ***(4)***. Primers are annealed *in situ* to complementary DNA targets on denaturated cell preparations, and then act as primers for chain elongation catalyzed by a *Taq* DNA polymerase in the presence of free nucleotides. The visualization of generated fragments results from the *in situ* incorporation of one labeled nucleotide.

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In the conventional PRINS procedure, the identification of several chromosomes is performed by *in situ* sequential labeling of each targeted chromosome, using chromosome-specific primers and different reporter molecules or fluorochromes. Between each labeling reaction, a intermediate reaction based on the use of ddNTP is performed to block the free 3'-ends of the generated elongation fragments, and then to prevent the mixing of the labeling ***(5)***. This intermediate reaction significantly extends the total duration of the multi-PRINS procedure and may lead to a prejudicial decrease in the intensity of the labeling of the first targeted chromosomes.

Recently, a new multicolor PRINS method has been introduced ***(6)*** that eliminates the blocking reaction. This original procedure, based on the *in situ* mixing of two fluorochromes for generating the distinct labeling of three chromosomes, greatly simplifies the multicolor PRINS protocol and increases the homogeneity of the chromosome labeling. We have adapted and tested this new PRINS protocol on isolated human oocytes and blastomeres.

2. Materials

2.1. Human Oocyte and Blastomere Preparation

1. Phosphate-buffered saline (PBS; Gibco BRL, Eragny, France).
2. Methanol, 99% (Prolabo, Paris, France).
3. Ethanol series: 70%, 90%, and 100% (Prolabo).
4. Glacial acetic acid (Prolabo).
5. Desionized formamide, stored at 4°C (Intergen Company, New York, NY).
6. 20X standard saline citrate (SSC): 3 *M* NaCl, 0.3 *M* trisodium citrate, pH 7.5 (can be stored for several months at room temperature).
7. Bovine serum albumin (BSA; Sigma, St. Louis, MO).
8. Distilled water.
9. 1 *N* HCl solution: dilute 1 mL of pure HCl (Prolabo) with 11 mL of distilled water. Store at 4°C.
10. Hypotonic solution: 1% Na citrate solution (Prolabo) in 0.2 mg/mL BSA.
11. Tween-20 (Roche Diagnostics).
12. Spreading solution: 0.01 *N* HCl, 0.1% Tween-20. Mix 1 mL of 1% Tween-20 with 0.1 mL of 1 *N* HCl solution and 8.9 mL of distilled water. Make spreading solution fresh.
13. Pepsine (Sigma): prepare a 10 mg/mL pepsine solution. Dilute 100 mg of pepsine in 10 mL of distilled water. Store frozen in 0.5-mL aliquots. Do not refreeze and only store for 1 to 2 mo.
14. Poly-L-lysine (Sigma).
15. Twin-frost glass microscope slides (CML, Nemours, France). The slides must be cleaned by soaking in absolute ethanol to which concentrated HCL have been added at the rate of 1 mL/100 mL. The slides are removed from the acid/alcohol and polished with clean piece of muslin just before dropping the sperm suspension.

16. Poly-L-lysine-coated slides: dilute 1:10 poly-L-lysine in water, pour into a Coplin jar, and incubate glass microscope slides for 5 min. Leave slides to dry at room temperature overnight and store in fridge.
17. Coplin jar (50 mL).
18. 25- to 50-µL micropipets (Drummond, Broomall, PA) attached to rubber tubing and mouthpiece.
19. 10- to 20-µL automatic pipetor.
20. Diamond marker.
21. Water bath at 37°C and 73°C.
22. Phase contrast microscope with ×10, ×40, ×60 magnifications (Leica France, Rueil-Malmaison, France).
23. Dissecting microscope equipped with a zoom system (Leica).

2.2. Multicolor PRINS

1. dATP: 100 m*M* solution (Roche Diagnostics, Meylan, France) diluted 1:10 with sterile distilled H_2O.
2. dCTP: 100 m*M* solution (Roche Diagnostics) diluted 1:10 with sterile distilled H_2O.
3. dGTP: 100 m*M* solution (Roche Diagnostics) diluted 1:10 with sterile distilled H_2O.
4. dTTP: 100 m*M* solution (Roche Diagnostics) diluted 1:100 with sterile distilled H_2O.
5. Fluorescein isothiocyanate 2'-deoxyuridine 5'-triphosphate (FITC-12-dUTP) 1 m*M* (Roche Diagnostics).
6. Tetramethylrhodamine isothiocyanate (TRITC-6-dUTP) 1 m*M* (Roche Diagnostics).
7. Cascade Blue-7-dUTP 1 m*M* (Molecular Probes, Leiden, The Netherlands).
8. 1X PBS.
9. BSA (Sigma, St. Louis, MO).
10. *Taq* DNA polymerase (Roche Diagnostics) or Ampli*Taq* (Perkin Elmer, Foster City, CA).
11. 10X *Taq* buffer: 500 m*M* KCl, 100 m*M* Tris-HCl, pH 8.3, 15 m*M* $MgCl_2$.
12. Oligonucleotide primers at 50 pmol/µL (*see* Table 1, Chapter 6).
13. Sterile distilled water.
14. Tween-20 (Roche Diagnostics).
15. Washing buffer (diluted from 20X SSC): 4X SSC, 0.05% Tween-20.
16. 1.5-mL Sterile microcentrifuge tubes (Eppendorf AG, Hamburg, Germany).
17. Cover slips (22 × 32 mm) (CML).
18. Coplin jar (50 mL).
19. Programmable thermal cycler equipped with a flat pate block (Hybaid Ltd., Teddington, UK).

2.3. Detection and Microscopy

1. DAPI (Sigma).
2. Propidium iodide (Sigma).
3. Antifade solution Vectashield (Vector Labs, Burlingame, CA).
4. Cover slips (20 × 40 mm; CML).
5. Rubber cement (Artos, Strasbourg, France).

6. Epifluorescence Microscope Leica DMRB (Leica France) equipped with ×40 and ×100 Plan FluoTar objectives, and with a DAPI single band-pass filter (Leitz filter A, cat. no. 513804), a FITC single band-pass filter (filter I3, cat. no. 513808), a TRITC single band-pass filter (filter N2.1, cat. no. 513812), a FITC/TRITC double band-pass filter (filter G/R, cat. no. 513803), and a triple filter (filter B/G/R, cat. no. 513836) for simultaneous observation of DAPI/Cascade-Blue, FITC and TRITC signals.
7. For image capturing, we use the software Metasystem Isis Version 5.0 (Metasystem, Altlusshein, Germany).

3. Methods

3.1. Human Oocyte and Blastomere Preparation

To develop these protocols, human oocytes and blatomeres are obtained after informed consent from women participating in in vitro fertilization (IVF) programs. The oocytes used are those that fail to fertilize and to cleave after in vitro insemination. Embryos used are donated embryo that have abnormal development and are not involved in IVF procedure. Single blastomeres are obtained from biopsy or by disaggregation of eight-cell-stage human embryo. Isolated oocytes and blastomeres are kept in drops of medium covered with silicone oil, in culture Petri dishes stored into an incubator at 37°C, 5% CO_2, and 95% humidity.

3.1.1. Human Oocyte Preparation

1. Using a micropipet, transfer individual oocyte from the culture medium droplet to hypotonic solution, under the control of a dissecting microscope (*see* **Note 1**).
2. The cell is exposed to hypotonic solution for approx 3 to 5 min under the control of the dissecting microscope. Check cell to make sure it has swollen 1.5 to 2 times its original size.
3. Carefully transfer the cell onto a microscopic slide in a 1- to 2-µL drop of hypotonic solution.
4. Using an automatic pipettor set at 17 µL, drop one drop of fresh fixative directly on citrate drop from a height of approx 2 cm (*see* **Note 2**).
5. As the drop starts to spread out on the slide, the cell will become quite visible under the fluorescent light, standing out in relief. A very gentle moist breath can be used to stop cell from rolling around the slide. This will add moisture to the slide and stabilize the cell. Use then a diamond marker to circle the cell on the underside of the slide.
6. Immediately add a second drop of fixative above the area where the cell was circled. *Do not allow the slide to dry*. The drop will spread out along the slide toward the edges and begin beading (*see* **Note 3**).
7. Add a third drop of fixative onto the cell. Do not allow the slide to dry.
8. When rainbow effect appears, add a fourth drop of fixative.

9. At this point, when the drop beading stops and the rainbow effect appears, use a long, gentle dry breath to dry the last fixative from the slide.
10. Using phase contrast microscope, check within the circle to ensure that nucleus or chromosomes are present.
11. Dehydrate the slide in an ethanol series (70%, 90%, 100%), 2 min each step, and air-dry.
12. Store the slide at room temperature in a hermetic box in order to avoid dust deposits.
13. Before the PRINS reaction, denature chromosomal DNA by immersing the slide in 70% formamide, 2X SSC, pH 7.0, at 73°C for 3 min.
14. Pass the slide through an ice-cold ethanol series (70%, 90%, 100%), 2 min each step, and air-dry.

3.1.2. Human Blastomere Preparation

It is possible to fix isolated blastomeres by using the above described fixation technique.

(**Subheading 3.1.1.**, **steps 1** to **12**). As an alternative, the following method can also be used (*see* **Note 4**):

1. Prepare fresh spreading solution by mixing 1 mL of 1% Tween-20 solution, 0.1 mL of 1 *N* HCl, and 8.9 mL of distilled water
2. Make a circle on the underside of a poly-L-lysine-coated slide using a diamond marker. Put a small drop of hypotonic solution on the slide in the circle and a small drop of 1X PBS in one corner of the slide.
3. Using a micropipet, transfer the isolated blastomere from the culture medium drop to the drop of PBS on the slide, under the control of a dissecting microscope.
4. Using a micropipet, transfer the cell into the drop of hypotonic solution within the circle, ensuring minimum transfer of PBS.
5. Before complete drying of the drop (approx 1–2 min), add continuously spreading solution to the drop until the cell starts to lyse (*see* **Note 5**). With constant observation of the cell, keep replacing the spreading solution until the lysis of the cell membrane. *The spreading solution must not be allowed to dry out before the complete cell lysis.*
7. When the cell is lysed and the cytoplasm washed away from the nucleus, leave the slide to air-dry and incubate in 1X PBS for 5 min.
8. Dehydrate the slide in an ethanol series (70%, 90%, 100%), 2 min each step, and air-dry.
9. At this stage, the slide can be stored for up to 1 wk at room temperature in a hermetic box (to avoid dust deposits).
10. Before the PRINS reaction, prepare a 0.01 *N* HCl solution in a Coplin jar and heat to 37°C in water bath. Add a 0.5-mL aliquot of 10 mg/mM pepsin to HCl and mix well.
11. Place the slide in this 1% pepsine solution and incubated the slide at 37°C for 3 to 10 min, depending on the amount of remaining cytoplasm (*see* **Note 6**).

12. Rinse briefly the slide on 1X PBS.
13. Wash the slide twice 1 min in distilled water.
14. Pass the slide through an ethanol series (70%, 90%, 100%), 2 min each step, and air-dry.
15. Denature chromosomal DNA by immersing the slide in 70% formamide; 2X SSC, pH 7.0; at 73°C for 3 min.
16. Pass the slide through an ice-cold ethanol series (70%, 90%, 100%), 2 min each step, and air-dry.

3.2. Multicolor PRINS (on Oocyte or Blastomere Preparations)

In the three-color PRINS procedure, three sequential PRINS reactions are performed, each labeling one specific chromosome. The following labeling order is used (*see* **Note 7**): (1) FITC for the first targeted chromosome, 92) TRITC for the second targeted chromosome, and (3) FITC for the third targeted chromosome. In the four-color procedure, this labeling order is supplemented with a fourth PRINS reaction using Cascade Blue (*see* **Fig. 1**, Chapter 6).

3.2.1. Three-Color PRINS Procedure

1. Prepare a reaction mixture for the first PRINS reaction, in a final volume of 50 µL containing: 0.2 m*M* dATP, dCTP, and dGTP, 0.02 m*M* dTTP, 0.02 m*M* FITC-12-dUTP, 50 m*M* KCl, 10 m*M* Tris-HCl, pH 8.3, 1.5 m*M* $MgCl_2$, 0.01% BSA, 200 pmol of oligonucleotide primer, and 2.5 U *Taq* DNA polymerase. In practice, mix in a sterile microcentrifuge tube: 1 µL of each 1:10 diluted dATP, dCTP, and dGTP, 1 µL of the 1:100 diluted dTTP, 1 µL of FITC-12-dUTP, 1 µL of BSA, 5 µL of 10X *Taq* buffer, 0.5 µL of the *Taq* DNA polymerase, 4 µL of the specific primer (for instance, the primer specific for chromosome 1 according to the procedure illustrated in **Fig. 1**, Chapter 6), and distilled water to 50 µL.
2. Place the reaction mixture under a 22 × 32 cover slip on the denatured slide, and transfer to the heating block of the thermal cycler.
3. Set up the PRINS program and start the reaction. The program consists of a unique 5- to 10-min step at the specific annealing temperature of the primer involved for both *in situ* annealing and elongation.
4. While this first reaction is running, prepare the reaction mixture for the second PRINS reaction as described previously but instead incorporate the specific primer for the second targeted chromosome (for instance, chromosome 9 according to **Fig. 1**, Chapter 6), and TRITC-6-dUTP.
5. On completion of the program, carefully remove the cover slip from the slide.
6. Wash the slide twice for 2 min at room temperature in 1X PBS.
7. After draining the excess 1X PBS off the slide, and before the slide is completely dry, put the second PRINS reaction mixture on the slide, and cover with a 22 × 32 cover slip.
8. Place the side again on the plate of the thermal cycler.

9. Set up the program for the second PRINS reaction: 5 min at the annealing temperature, specific to the second primer used.
10. Start the program.

No additional denaturation is required after the first PRINS reaction because DNA remains denatured through the PRINS incubations.

11. While this second reaction is running, prepare the reaction mixture for the third PRINS reaction, incorporating the specific primer for the third targeted chromosome (for instance chromosome 16 according to **Fig. 1**, Chapter 6) and FITC-12-dUTP.
12. At the end of the second reaction, remove the cover slip from the slide and repeat the washing **steps 6** and **7**.
13. Before the slide is completely dry, put the third PRINS reaction mixture on the slide, and cover with a 22 × 32 cover slip.
14. Place the slide on the thermal cycler.
15. Set up the program for the third PRINS reaction: 5 min at the annealing temperature, specific to the third primer used.
16. Start the program.
17. At the end of this third reaction, the slide is transferred to 4X SSC, 0.05 Tween-20 for two washes (3 min each) at room temperature, with gentle agitation.

3.2.2. Four-Color PRINS Procedure

In the four-color procedure, the third reaction is followed by a fourth reaction with the primer specific for the fourth targeted chromosome (for instance chromosome 18 as indicated in **Fig. 1**, Chapter 6). No additional denaturation is needed.

1. Prepare the reaction mixture for the fourth PRINS reaction, incorporating the specific primer for the fourth targeted chromosome and Cascade Blue-7-dUTP.
2. At the end of the reaction, remove the cover slip from the slide.
3. Transfer the slide in 4X SSC, 0.05 Tween-20 for two washes (3 min each) at room temperature, with gentle agitation.

3.3. Detection and Microscopy

1. Drain the excess washing solution off the slide.
2. Mount the slide in Vectashield antifade solution containing either DAPI (0.3 µL/mL) or a mix of propidium iodide (0.3 µL/mL) and DAPI (0.3 µL/mL).
3. Cover with a 22 × 40-mm cover slip and seal the cover slip with rubber cement.
4. Examine the slide under the epifluorescence microscope, preferentially using first the triple or double band-pass filter, and confirming the coloration of the fluorescent spot with single band-pass filters. **Figure 1** shows an example of PRINS labeling performed on human oocyte chromosomes.

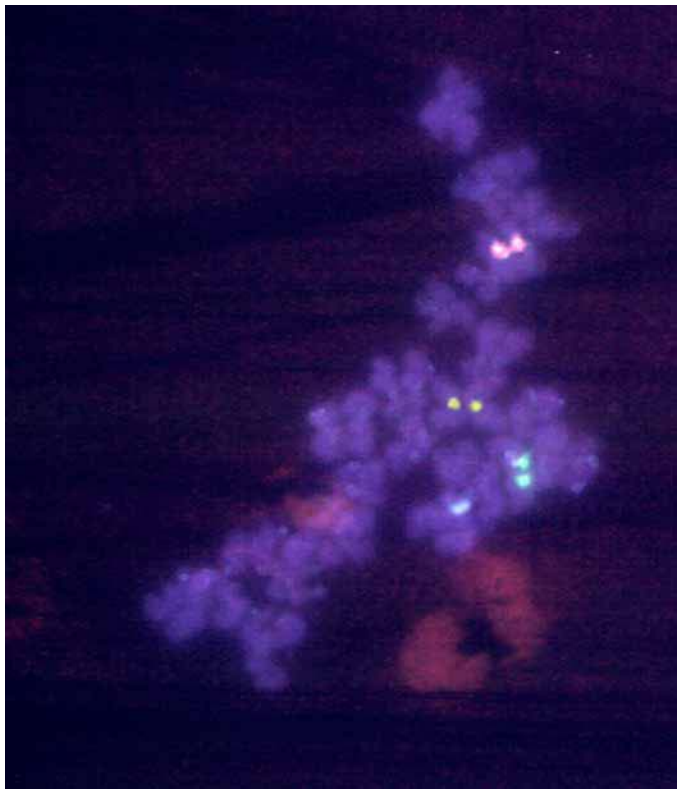

Fig. 1. Four-color PRINS labeling of human oocyte chromosomes using the labeling order FITC/TRITC/FITC/Cascade Blue. Chromosome 1 is labeled in yellow, chromosome 7 in green, chromosome 9 in red, and chromosome 16 in blue. (Please *see* color insert following p. 48.)

4. Notes

1. Make sure that the pipet is free of oil inside and outside. Excess oil must be removed from the pipet to avoid the formation of an oil layer on the solution surface. The presence of oil on the slide interferes with the proper fixation of the cell. Two watch dishes can be used to prevent the transfer of oil to the slide.
2. The fixation is the key to the success of the entire technique. Fixation removes the cytoplasm material and makes the nuclear DNA accessible to subsequent labeling procedure. Relative humidity is essential in the area where cell fixation takes place. A relative humidity of 40 to 50% must be maintained. It is also important that standard fluorescent lighting is available above the area where fixation will perform so that egg can be observed by the reflection of the light and solution spreading on the slide can be observed.
3. At this point, a rainbow formation visible under the standard ceiling fluorescent lighting will begin moving in toward the center of the slide where the cell is.

Allow the rainbow to come within 0.5 cm on either side of where the egg is before dropping a new drop of fixative.

4. Blastomere fixation was initially performed using classical Tarkowsky technique using methanol/acetic acid as a fixative agent. After the publication of the HCl-Tween-20 technique ***(7)***, some laboratories began using this alternative procedure, which allows to monitor the lysis of the cell and limits scattering of nuclear material.
5. First, the cell membrane is ruptured and the nucleus is separated from the bulk of the cytoplasm. The shape of the blastomere may distort and at this stage, the nucleus should be visible. Keep watching the nucleus. The nucleus can be obscured by the addition of too much spreading solution and lost from inconsistent observation. If at any point you lose sight of it, stop and let the slide dry.
6. Pepsine is used to remove excess cytoplasmic proteins on the slide. The removal of cytoplasmic remnants is critical. If the pepsin treatment is not sufficient, the remaining cytoplasmic proteins will prevent the binding of probes or primers. On the other hand, overexposure to pepsin may lead to the degradation of targeted DNA. Be careful because the pepsine activity can vary from batch to batch.
7. For the three-color PRINS reactions, the combination order FITC/TRITC/FITC give the best results, with well distinct red, green, and yellow spots. When using the reversed labeling combination TRITC/FITC/TRITC, no pure yellow color, but a mixed orange color is obtained for the first labeled chromosome. Indeed, a mean ratio of green to red color of 70%:30% must be respected to obtain well-defined green, red, and yellow signals. In the four-color procedure, the addition of the blue label does not affect the final coloring of the three previously labeled targets. The Cascade Blue dye provides a color which contrasts well with the longer-wavelength green and red fluorophores.

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16

Microdissection With PCR *In Situ*

Allen T. Christian and Christine Hara

Summary

In situ amplification techniques are designed to increase the mass of DNA in a fixed target, either whole cells or tissue sections. When combined with fluorescently labeled nucleotides, they can be used for locus detection. They also can be used to increase target mass for subsequent operations, such as cellular or chromosomal isolation by microdissection. When combined with chromosome microdissection, these techniques allow libraries to be made from single copies of chromosomes, chromosome fragments, or even bacteria.

Key Words: PCR *in situ*; whole genome amplification; *in situ* hybridization; FISH, microdissection.

1. Introduction

Microdissection is an extremely useful method of precisely isolating chromosomes, chromosome fragments, and bacteria ***(1–3)***. With few exceptions, microdissection has required that multiple copies of a desired template be isolated, to obtain sufficient material for subsequent amplification. This is frequently possible, as in the case of G-banded human chromosomes. However, for many case, such as isolated fragments, specific bands, and derivative chromosomes, it is advantageous to be able to amplify a DNA library made from a single template. For this purpose, preamplifying the template before microdissection can yield excellent results.

In situ applications of polymerase chain reaction (PCR) are used primarily to detect targets too small to be seen with traditional *in situ* hybridization techniques ***(4)***. In certain cases, they also can provide increased specificity and more rapid analyses. In addition to target location, however, PCR *in situ* and other genome amplification techniques can be used to increase the total amount of DNA associated with a genome or with a specific region of a genome ***(5,6)***.

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The techniques described in this chapter can be used in either eukaryotic or prokaryotic cells and are described to enable results directly from following the protocols but can be generalized to allow them to be adapted to specific requirements.

2. Materials

2.1. Slide Preparation

1. Fixative: 3:1 methanol:glacial acetic acid.
2. Methanol 100%.
3. 0.075 KCl hypotonic solution.
4. Centrifuge.
5. Falcon tube.
6. Microscope slide.

2.2. Degenerative Oligonucleotide-Primed PCR In Situ

1. Template cover slip.
2. Thermo Sequenase DNA Polymerase, Thermo Sequenase reaction buffer (Amersham, Arlington Heights, IL). Aliquot upon arrival and store at –20°C until needed (*see* **Note 1**).
3. dNTP mix (Roche, Indianapolis, IN): Dilute each dNTP dATP, dCTP, dGTP, and dTTP, each at a stock concentration of 100 m*M*) in dH_2O to a working concentration of 2 m*M* per dNTP. Aliquot and store at –20°C.
4. Tetramethylrhodamine-6-dUTP, stock 1 m*M* (Molecular Probes, Eugene, OR). Dilute to 40 µ*M* and aliquot. Store at –20°C (*see* **Note 2**).
5. Degenerative oligonucleotide-primed primer (5'CCGACTCGAGNNNNNN ATGTGG-3'). Resuspend with 1X Tris-EDTA buffer, pH 8.0, to a stock concentration of 40 µ*M*. Aliquot and store at –20°C.
6. 20X Standard saline citrate (SSC): Thoroughly dissolve 174.3 g of NaCl and 88.2 g of trisodium citrate in 800 mL of double distilled H_2O. Bring to a final volume of 1 L with ddH_2O and adjust the pH to 5.3 with concentrated HCl. Adjusting the pH of the 20X stock to 5.3 will produce a pH of 7.25 when diluted to a 2X SSC/70% formamide solution. Store at room temperature until needed.
7. 4X SSC, 0.1% Triton X-100: Dilute 20X SSC to a final concentration of 4X SSC, 0.1% Triton X-100 (by volume) by adding the appropriate amount of Triton X-100 and diluting with dH_2O. Store at room temperature.
8. Mounting medium: 0.3 µg/mL Vectashield with DAPI (Vector Laboratories Inc., Burlingame, CA).
9. Rubber cement. Store at room temperature.
10. Clean microscope slides.
11. Clean tissues.
12. Zeiss Axioskop (Carl Zeiss, Inc, Thornwood, NY) and images captured by a Vysis QUIPS Imaging Analysis System (Vysis, Downers Grove, IL).

2.3. Whole Genome Amplification

1. Template cover slip. Template cover slips should be used for subsequent microdissection.
2. 20X SSC: Adjust the pH to 5.3 with concentrated HCl.
3. 0% Formamide, 2X SSC: Dilute 70 mL of formamide and 10 mL of 20X SSC, pH 5.3, with 20 mL of H_2O for a 100-mL total volume. Seal container with parafilm and store at 4°C until ready for use. Heat to 70°C in a Coplin jar for each experiment. Make fresh for each experiment.
4. Ethanol 70%, 85%, and 100% room temperature: Dilute 200 proof ethanol with distilled (d)H_2O to 70%, 85%, 50 mL per percent. Store stock at room temperature in a flammable cabinet when not in use.
5. phi29 DNA Polymerase, 10,000 U/mL (NEB, Beverly, MA). Aliquot upon arrival and store at –20°C.
6. 10X phi29 DNA Polymerase buffer: 10X buffer is provided with phi29 DNA Polymerase. Aliquot and store at –20°C upon arrival. 1X buffer is prepared fresh for each experiment by diluting 10 µL of 10X buffer and 2 µL of 10 mg/mL bovine serum albumin in 88 µL of dH_2O (*see* **Note 3**).
7. dNTP mix: (Roche): Dilute each dNTP (dATP, dCTP, dGTP, dTTP, each at a stock concentration of 100 m*M* each) in dH_2O to a working concentration of 2 m*M* per dNTP. Aliquot and store at –20°C.
8. 4 m*M* dithiothreitol (DTT): the DTT used for our experiments was obtained from the 1 *M* stock vial provided with the Molecular Staging Repli-g kit and diluted with sterile dH_2O to 4m*M*. However, this DTT was used by default. DTT purchased from a vendor will be more than adequate.
9. 2 m*M* Random hexamers, 5'-nnnnnn-3' where *n* = A, T, C, or G (Genosys, Woodlands, TX). Primers will arrive dry and will need to be resuspended to 2 m*M* with 1X Tris-EDTA buffer, pH 8.0 (Invitrogen, Carlsbad, CA).
10. Clean 24 × 60-mm cover slips.

2.4. Microdissection

It should be noted that we describe only micromanipulator-based microdissection. Laser capture microdissection, a powerful technique, is more often used in tissue isolation, and is less frequently used to isolate such small-mass items as chromosomes.

1. Inverted microscope (Zeiss Axiovert); an upright microscope is unsuitable because the glass needle will not fit between the objective and the cover slip. It should have phase contrast, and minimum two objectives: ×10 and ×100. It is unnecessary to have any image analysis capability. However, a free rotating stage is quite useful because it allows the chromosome to be oriented in a particular way.
2. Needles (Frederick Haer & Co.). These can be made or purchased. For simple, frequent chromosome microdissection, making them by pulling capillary tubes

(1.0 mm OD × 0.75 mm ID or 1.0 mm OD × 0.5 mm ID [both 100 mm in length] work well for dissecting chromosomes, and eukaryotic and prokaryotic cells) is the least expensive option. In the event more complex operations (such as microinjection) need to be undertaken, or if microdissection will be an infrequent operation, they can be purchased (e.g., from Eppendorf).

3. Needle puller (Sutter Instruments model P-30). It is unnecessary to use one with the full capabilities of making patch clamp needles; the simplest model extant is sufficient.
4. Micromanipulator (Eppendorf model 5171). There are several possibilities depending on the level of precision desired. Narashige and Eppendorf make excellent micromanipulators, over a wide array of cost. All are sufficient for the task, but spending more is helpful in that more capabilities, such as automatically returning to a metaphase spread after replacing a needle, are offered.

3. Methods

3.1. Slide Preparation

1. Soak cover slips in 100% methanol for 5 min to remove any coating or grime.
2. Remove and dry with a lint-free towel.
3. Store in a clean, dry place until needed.

3.2. Bacterial Cultures

1. Grow desired bacterial cell line in the proper liquid medium overnight at 37°C, approx 230 rpm.
2. Place a drop (approx 2 µL) of liquid culture onto opposing ends of a clean slide. Add a drop of 3:1 methanol:glacial acetic acid to each of these areas. Heat at 37°C until dry. Set slides aside until needed.

3.3. Cell Cultures

1. Grow desired cell line in the appropriate growth medium at 37°C, 5% CO_2.
2. For metaphase cells: add 0.1 µg/mL of culture colcemid to the flask 4 h before harvesting (*see* **Note 4**). After incubation lift cells from flask and place in 15-mL Falcon tube.
3. Centrifuge the liquid culture for 5 min at 150*g* in a benchtop centrifuge. Repeat this step until the supernatant is clear. Carefully remove it and discard. Add 8 mL of 0.075 KCl hypotonic solution to each falcon tube and gently resuspend using a plastic pipet. Recap the Falcon tubes. Set a timer for 20 min for Chinese hamster ovary cells, 30 min for rat and human lymphocytes/lymphoblastoid cells, or 60 min for mouse lymphocytes. Incubate cells in solution at 37°C.
4. When hypotonic is complete, add 1 mL of fixative. Mix gently and centrifuge for 5 min at 150*g*.
5. Remove supernatant, add 3 mL of fixative and gently resuspend. Repeat this process until the fixative supernatant is colorless.

6. Centrifuge at 150*g* for 5 min one last time and resuspend the pellet in 10 drops of fix if the pellet is large or 5 drops if the pellet is small. The cell/fix mixture should be slightly cloudy.
7. Place two drops of cell/fix mixture on opposing ends of a clean slide. Add one more drop of fix on top of the cells and let air-dry.

3.4. DOP-PCR In Situ

1. To detect positive amplification, use two template cover slips for DOP-PCR *in situ*. One will be used as a positive control by incorporating rhodamine-6-dUTP and the other will be used for microdissection and subsequent probe paint.
2. The reaction mixture has a 50-µL total volume and consists of 25 U Thermo Sequenase DNA Polymerase, 5 µL of Thermo Sequenase reaction buffer, 200 µ*M* of each dATP, dTTP, dCTP, and dGTP, and 4 µ*M* of DOP primer (5'-CCGACTCGAGNNNNNNATGTGG-3'). Control DOP-PCR *in situ* experiments included 40 µ*M* tetramethylrhodamine-6-dUTP added to the reaction in addition to the other components. Place each reaction drop onto unfrosted microscope slides.
3. Carefully invert the template cover slip onto the slide with the reaction mixture so that the cells are immersed in solution.
4. Seal the edges with rubber cement. Carefully examine the seal for air bubbles after drying. Additional rubber cement should be administered if bubbles have formed (*see* **Note 5**).
5. Place the slides on a thermal cycler and without a heated lid use the following thermal profile; 95°C for 10 min, 8 cycles at 94°C for 1 min, 30°C for 5 min, and a ramp of 0.1°C/s up to 65°C for 5 min, 12 cycles at 94°C for 1 min, 56°C for 5 min, and 72°C for 5 min, followed by 72°C for 5 min and hold at 4°C until the slides can be removed (*see* **Notes 6**–**8**).
6. Carefully remove the cover slips with a razorblade or other sharp edge, and soak them in a 4X SSC, 0.1% triton X-100 solution for 5 min at room temperature.
7. Mount the fluorochrome labeled cover slip onto a microscope slide using the mounting medium (approx 10 µL for a 22 × 22-mm cover slip).
8. Blot away excess medium with a clean tissue.
9. Metaphase spreads can be visualized using a Zeiss Axioskop (Carl Zeiss, Inc) and images captured by a Vysis QUIPS Imaging Analysis System (Vysis).
10. PCR is considered successful if all choromosomes in a metaphase spread are labeled with fluorochrome.

3.5. Whole-Genome Amplification

1. Because of the sensitivity of whole-genome amplification, reaction set-up should be performed in a sterile hood. Sterile sleeves should be worn at all times, and great care must be taken to avoid contamination.
2. Template cover slips or slides must be denatured before amplification.
3. Denature the cover slips in 70% formamide, 2X SSC for 5 min at 70°C. Remove and run through the ethanol series: 70%, 85%, and 100% for 2 min each.

4. Remove and set aside to air-dry.
5. Whole-genome amplification is performed by mixing 10 µL of 2 m*M* dNTP, 8 µL of 1X phi buffer mix, 2 µL of 4 m*M* DTT, 2 µL of 2 m*M* random hexamers, and 1.0 µL of phi29 DNA Polymerase per reaction.
6. Transfer the 21-µL reaction mixture onto the template cover slip and cover with a clean microscope slide.
7. Seal the edges with rubber cement. Carefully examine the seal for air bubbles. After drying air bubbles may have formed. Additional rubber cement should be administered if air bubbles are present.
8. Incubate on a thermal cycler, without a heated lid, at 30°C for the desired amount of time (4 h may be sufficient for subsequent microdissection, but amplification may occur for as long as 16 h. A larger reaction volume may be necessary for 16-h incubation owing to evaporation).
9. Heat-denature the polymerase at 65°C for 10 min.
10. Lower temperature to 4°C to chill.
11. Carefully remove the cover slip and set aside for subsequent microdissection.

3.6. Microdissection

1. Place the glass pipet in the micropipet puller and pull needles to the desired tip width (*see* **Note 9**).
2. Place template cover slip face up onto the stage of the inverted microscope (*see* **Note 10**).
3. Place needle in the micromanipulator at angle of less than 45°. Needles may be broken by the cover slip if their resting angle is too steep. If the angle is too steep, it is hard to pick up dissected fragments; too low, and it is hard to cut fragments.
4. Move the needle tip directly to the center of the field of view; approx 1 cm above the stage and template cover slip.
5. Lower the focal plane slightly, then lower the needle until it is in focus. Continue this process until the needle is just above the cover slip surface. This is preferable to lowering the needle and then focusing on it, which will generally result in the needle hitting the cover slip and breaking. Once the needle is just above the cover slip, rotate the objectives to the ×100 lens.
6. At this point, the micromanipulator should be switched from coarse to fine motion. This process varies depending on the brand of micromanipulator, but if such a dual control system is present, the fine control makes movement at ×100 much easier.
7. The needle should now be lowered slowly until it ceases moving down and moves slightly to the left. At this point, it should be moved to the left with the left-adjusting control of the micromanipulator, to scrape the chromosome (In a typical set-up, the needle will be coming from the right, pointing to the left.) Select your target, and orient it so that the needle will cut in the appropriate direction.
8. In a scooping motion slowly move the tip of the needle down and at an angle under the cut fragment, or the whole chromosome or bacteria, carefully trying to get below the object. When the needle is located underneath the object, continue

moving it laterally while slowly moving it up and away from the stage. The proper motion is a bit like an airplane taking off. Once the fragment is on the needle and off the surface, it is very unlikely to come off of the needle.

9. When the needle tip is a safe working distance from the stage (approx 1 cm), carefully remove the needle from the micromanipulator.
10. Carefully break off the tip of the needle into a dry, sterile 0.2-mL thin-walled microfuge tube for subsequent reactions.
11. Add the PCR master mix to the wall of the tube after the fragment(s) are collected, cap the tube and centrifuge to get everything to the bottom of the tube, and thermocycle as described in **Subheading 3.4.**, **step 5**.

4. Notes

1. Polymerase choice is important; one must use a polymerase that is reasonably unimpeded by chromosome structure. We find that Thermo Sequenase works well, but there are others that we have not used, which may work as well or better. Experimentation may be useful.
2. A fluorescent nucleotide is unnecessary to the processes of DOP-PCR *in situ* and whole-genome amplification but is useful as a control to assess efficacy of the reaction. Typically, it is used in parallel with an unlabeled reaction; the products of the unlabeled reaction are used for subsequent amplifications.
3. The choice of polymerase is equally important for WGA. The ϕ29 bacteriophage DNA polymerase has an extremely high processivity and will produce large quantities of accurate product. It is, however, not thermostable, and must not be present during any denaturation steps.
4. This time can be varied according to many circumstances. The longer the colcemid is left on the cells, the more condensed the metaphase chromosomes will become, which is advantageous for microdissecting whole chromosomes because it is easier to pick up condensed chromosomes. For isolating bands, it is much easier to use less condensed chromosomes. Colcemid times also depend on the fraction of mitotics desired and the division time of the individual cells. Chinese hamster ovary cells double in approx 12 h, half the time of human cells. To obtain a vary high fraction of mitotic human cells, we frequently leave the colcemid on the cultures for as long as 30 h.
5. Any gap in the rubber cement will allow the mix on the slide to evaporate. It is critical to have a well-sealed cover slip. Equally important is removing all air bubbles from between the slide and cover slip before applying the rubber cement, as they will expand and force out the liquid during heating. Gently lifting and lowering one corner of the cover slip with a razor blade or similar object will help remove the bubbles.
6. A flat block, dedicated to slides, is not necessary. Simply covering a normal microcentrifuge tube-capable block with aluminum foil is more than sufficient.
7. Humidity in the chamber is reasonably important because allowing the rubber cement to dry out can result in the PCR mix evaporating away during the reaction. It can occasionally be difficult to tell if this has happened by inspecting the slide, but very high backgrounds and spotty results are indicative of evaporation.

8. A primary issue with cycling PRINS and PCR *in situ* is product drift; if too many cycles are performed, fluorescently labeled product can leave the target locus and drift elsewhere. It is necessary to experiment to find the minimum number of cycles necessary to locate the desired locus, and not to perform more cycles than are absolutely necessary.
9. A handy needle holder consists of modeling clay flattened out in the bottom of an uncoated tissue culture plate; stick the unpointed end of each needle in the modeling clay and point straight up. The settings on the needle puller must be determined empirically, and depend on the type and thickness of capillary tube chosen, the puller itself, and the target to be isolated. For whole chromosomes and bacteria, a thicker needle is better. For chromosome bands or microchromosomes, a finer point is necessary.
10. This is a significant issue. It is impossible to tell on which side of the cover slip cells are without trying to scrape them with the needle. We have found that writing the word "cells" backward on the cover slip's noncell side works well. When the cover slip is held cell-side up, the word can be read. This will keep the ink off of the side on which reactions will take place.

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17

Application of Reverse Transcription *In Situ* PCR in Cancer Analysis

Tamotsu Takeuchi, Yoshihiro Adachi, Hiroshi Sonobe, and Yuji Ohtsuki

Summary

It is now well recognized that chromosomal translocation followed by overexpression of a chimeric gene product plays a critical role in tumorigenicity in various malignant tumors, especially those of leukemia, malignant lymphoma, and soft-tissue tumors. In these malignant tumors, specific chimeric gene products are directly related to tumorigenicity. Therefore, if chimeric gene products could be observed *in situ*, it would be advantageous not only for the correct diagnosis of each tumor but also to improve our understanding of the basis of tumorigenicity. Accordingly, it would seem that reverse transcriptase (RT) *in situ* polymerase chain reaction (PCR) is a powerful and useful approach for the study of chimeric gene products *in situ*. Here, we introduce the application of RT *in situ* PCR to detect a hybrid, SYT-SSX messenger RNA in synovial sarcoma. We expect that the principle of this protocol also may be applied to detect other chimeric gene products.

Key Words: RT *in situ* PCR; cancer; synovial sarcoma; SYT-SSX; diagnostic pathology; soft tissue tumor.

1. Introduction

In situ hybridization is a well-established method used to detect messenger RNA in tissue and is used widely. In contrast, reverse transcription (RT) *in situ* polymerase chain reaction (PCR ***[1,2]***) has remained in the developmental stage, and the technique requires further refining before it can be used for specific applications. Although many laboratories described applications of RT *in situ* PCR in various fields during the 1990s, there also has been considerable criticism regarding the method.

As shown in **Fig. 1**, there are several variations of RT *in situ* PCR In so-called “direct detection,” primers, which are used for PCR amplification,

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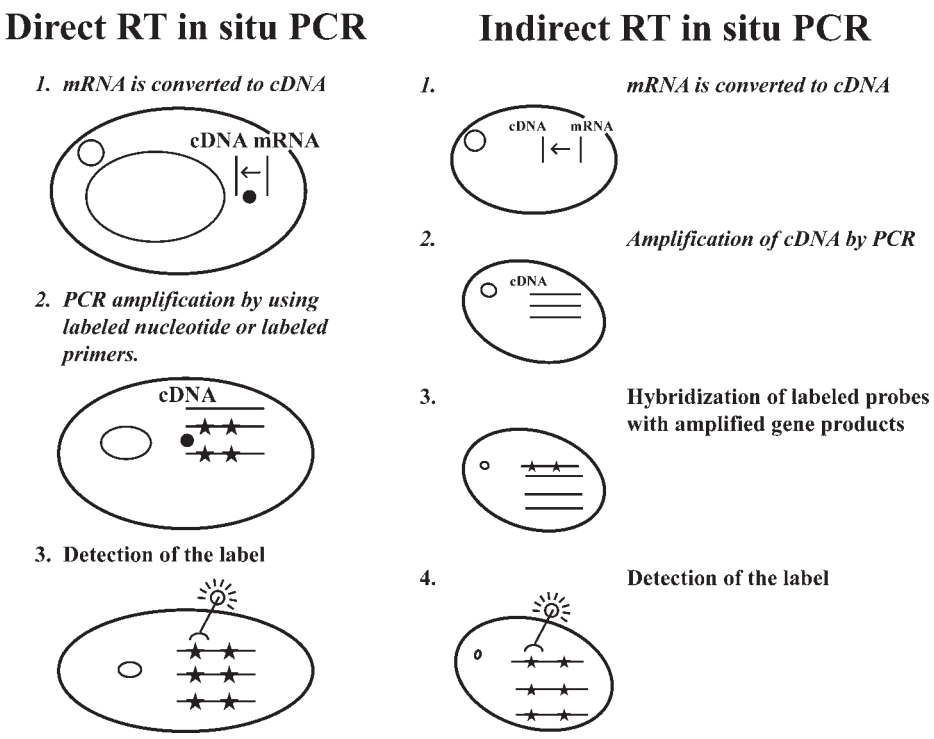

Fig. 1. Schematic representation of principal steps for direct or indirect RT *in situ* PCR. In both the direct and indirect methods, mRNA is first converted to cDNA (1, RT step). Subsequently, PCR amplification is performed by using the cDNA as a templates (2, PCR amplification step). In the direct method, amplified products harboring with labeled nucleotide or primers are detected after immunohistochemical staining (3, immunohistochemical detection step). By contrast, in the indirect method, amplified PCR products are hybridized with specific labeled primers (3, hybridization step), then detected by immunohistochemical staining (step 4).

are labeled with biotin, digoxigenin (DIG), or other fluorescein reagents. Alternatively, the labeled nucleotide, DIG-11-UTP (dTUP coupled with, DIG via an alkali-labile ester-bond) is incorporated into amplified products during PCR. In these direct methods, the labeled products can be visualized at the end of the reaction. In "indirect detection" ***(3)***, PCR is performed with unlabeled primers and dNTP in a similar manner to standard RT-PCR conducted in a tube and, subsequently, amplified products are hybridized with a labeled probe. Then, specifically hybridized probes are observed in a manner similar to that used in Southern or Northern hybridization.

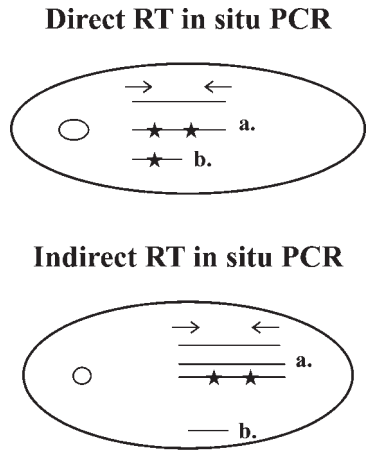

Fig. 2. Nonspecific amplification which may cause "false-positive" in direct *in situ* PCR method. It is possible to detect the nonspecific PCR products (indicated as b) by the direct RT *in situ* PCR, whereas the hybridization step could neglect the nonspecific products in indirect RT *in situ* PCR. Therefore, indirect RT *in situ* PCR could specifically detect the correct amplification products (a).

The direct method is shorter, less expensive, and minimizes the manipulation of the tissues if proper signals are obtained ***(4)***. However, compared with direct methods, there is an advantage in indirect RT *in situ* PCR with regard to specificity ***(3,5)***. As demonstrated in **Fig. 2**, we cannot discriminate the nonspecific signals from correct signals in direct RT *in situ* PCR It is not uncommon to observe nonspecific bands, which are of an undesired length, in standard RT-PCR. It also is not uncommon to observe several nonspecific bands or even smears in inappropriate PCR conditions. These phenomena can occur as the result of poor specificity of the primer sets used or excess quantities of *Taq* polymerase or $MgCl_2$. As described herein large quantities of *Taq* polymerase and $MgCl_2$ usually are used in RT *in situ* PCR, compared with standard RT-PCR, to increase the sensitivity. Therefore, we suggest that indirect RT *in situ* PCR more reliably excludes nonspecific amplification by using hybridization steps after PCR.

The application of indirect RT *in situ* PCR consists of three major steps. First, complementary (c)DNA is synthesized *in situ* from mRNA by reverse transcriptase. Second, synthesized cDNA is amplified with primers by *in situ*

PCR Third, amplified products are visualized by hybridization with labeled probes.

The three major problems in RT *in situ* PCR are (1) mRNA is easily degenerated by RNase, which can contaminate samples and originate from almost anywhere; (2) it is necessary to take controls to verify the results; and (3) in general, the sensitivity of RT *in situ* PCR is not as high as expected because of poor amplification of gene products, which is estimated to be just 100-fold in tissue sections.

With respect to the first problem, it is important to consider the degree degradation of mRNA before initiating the RT process. In the detection of murine mRNA by *in situ* hybridization, the tissues usually are fixed using circulation or immediately frozen followed by fixation. This careful handling of specimens may prevent the degradation of mRNA, which occurs during the delay between tissue isolation and fixation. In human tissues, the delay between tissue isolation and fixation typically is long and, thus, it often is difficult to obtain sufficient signal intensity in most routinely processed pathological tissues sections by *in situ* hybridization. Although the sensitivity of RT *in situ* PCR can be greater than simple *in situ* hybridization, we believe that inappropriately processed paraffin-embedded tissue sections are not suitable to detect mRNA *in situ.*

In the second problem, we have to discriminate true signals from fake signals, which are arising from hybridization with genomic DNA. We found this especially problematic when trying to detect viral RNA by RT *in situ* PCR However, we believe the same problem is less likely to occur in detecting chimeric genes by RT *in situ* PCR This is because the length of genomic DNA that represents chimeric mRNA is very long and is difficult to amplify by RT *in situ* PCR It generally is accepted that the limited length, which we detect by using paraffin-embedded tissue as a template, may be less than 500 to 600 base pairs in standard PCR ***(6)***. Furthermore, most tumors may also contain nonneoplasmic cells, including infiltrating leukocytes, which also may provide quality control for RT *in situ* PCR

In the third problem, the mechanism, which is responsible for the poor efficiency of RT *in situ* PCR, still remains obscure. However, the so-called "diffusion artifact" may be a major cause of this poor efficiency. During the PCR steps, the amplification products may not remain in the locality of the initial template. A significant proportion of amplified products may float into the reaction buffer and be used as template by subsequent PCR steps. Overlaying tissue sections with a thin layer of agarose may help to decrease the effects of the "diffusion artifact" ***(7)***; however, it may reduce the permeability required to allow polymerase to reach the template. We speculate that the reliability of RT *in situ* PCR would be greatly increased if the effects of the diffusion artifact could be minimized.

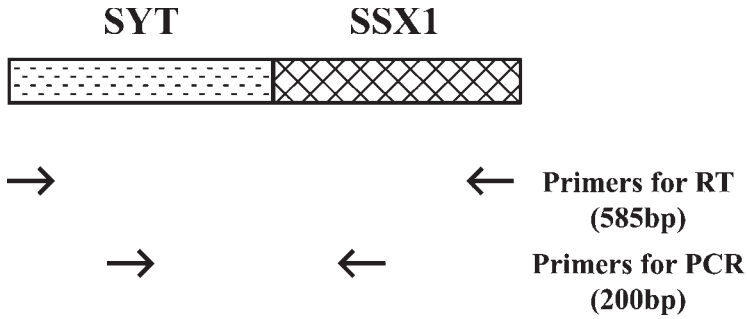

Fig. 3. Schematic representation of primers sets used in the current protocol.

Here, we describe our current protocol to detect a chimeric oncogene in human soft tumor, synovial sarcoma, by RT *in situ* PCR Synovial sarcomas are typified by a unique chromosomal translocation t(X;18)(p11.2;q11.2) that results in fusion of the *SYT* gene on chromosome 18 with the *SSX1* or *SSX2* gene in Xp11.2 and, consequently, production of the chimeric, SYT-SSX proteins (**Fig. 3** ***[8,9]***). It is suggested that SYT-SSX is involved directly in tumorigenicity of synovial sarcoma by interfering with the function of chromatin remodeling ***(10,11)***. Synovial sarcoma is not restricted to the synovium and can actually occur at any site. Unfortunately, histological diagnosis of synovial sarcoma, especially that of a monophasic fibrous type and/or in an extrasynovial site, can sometimes be difficult. As a result, it is desirable to establish dependable methods to reliably identify synovial sarcoma using pathologically processed specimens. Previously, we tried to detect chimeric, SYT-SSX mRNA by simple *in situ* hybridization; however, sufficient signals were not detected in almost all paraffin embedded specimens, which may have been attributable to insufficient quantities of SYT-SSX mRNA in synovial sarcoma cells.

2. Materials

2.1. Preparation of Tissue Sections (see Note 1)

1. MAS-coated precleaned Micro slide glass (Matsunami, Japan; *see* **Note 2**).
2. 10% Formalin neutral buffer solution (Wako Co. Ltd, Japan).
3. Histoprep 580, paraffin (Wako Co. Ltd).
4. Lemosol (Wako Co. Ltd).

2.2. Reverse Transcriptase

1. Proteinase K (RNase-free grade, Takara, Japan).
2. RNase-free DNase: 5 U RNase-free, DNase in 20 m*M* Tris-HCl, pH 7.4, 10 m*M* NaCl, 5 m*M* $MgCl_2$, 0.1 m*M* $CaCl_2$.
3. RNA long and accurate (LA) PCR kit (AMV) Ver. 1.1 (Takara; *see* **Note 3**).

4. Primers, sense: 5'-CAACAGCAAGATGCATACCA-3' (forward primer for SYT); antisense: 5'-CACTTGCTATGCACCTGATG-3' (reverse primer for SSX1).
5. RT reaction buffer: 10 m*M* Tris-HCl, 50m*M* KCl, 1.5 m*M* $MgCl_2$, 25m*M* dNTPs.
6. 100 n*M* SYT-SSX primer sets, 50 U RNase inhibitor, and 400 U Moloney murine leukemia virus reverse transcriptase.
7. Hybaid OmniSlide *In-Situ* Thermal Cycler (Hybaid Ltd, UK; *see* **Note 4**).
8. Hybaid EasiSeal (Hybaid Ltd).

2.3. Polymerase Chain Reaction

1. AmpliTaq, GOLD polymerase (Applied Biosystems, CA).
2. 10X PCR buffer: 100 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl, 0.01% (w/v) gelatin, dNTPs, and 25m*M* $MgCl_2$ solution are attached to this enzyme.
3. Hybaid OmniSlide *In-Situ* Thermal Cycler (Hybaid Ltd).
4. Hybaid EasySeal (Hybaid Ltd).
5. Primers. sense: 5'-GCTACGGTCCTTCACAGGGTGGTCCAGG-3' (nested primer for SYT); antisense: 5'-AGATGCTTCTGACACTCCCTTCGAATC-3' (nested primer for SSX1).
6. Washing solution: 10 m*M* Tris-HCl, pH 7.5.

2.4. Labeling and Hybridization of Probe

1. RNA LA PCR kit (AMV) Ver. 1.1 (Takara).
2. Trizol Reagent (GibcoBRL, Rockville, MD).
3. DIG high prime-labeling and detection kit (Boehringer Mannheim GmbH, Germany).
4. DIG Easy Hyb (Boehringer Mannheim GmbH).
5. 20X standard saline citrate (SSC): 3 *M* NaCl, 0.3 *M* sodium citrate (pH 7.4).
6. 2X SSC: Dilute 20X SSC 10-fold with distilled water.
7. 0.5X SSC: Dilute 20X SSC 40-fold with distilled water.

2.5. Immunohistchemical Detection of DIG

1. DIG high prime-labeling and detection kit (Boehringer Mannheim GmbH; *see* **Note 5**).
2. Maleic acid buffer: 0.1 *M* maleic acid, 0.15 *M* NaCl. Adjust with NaOH to pH 7.5.
3. Detection buffer: 0.1 *M* Tris-HCl, 0.1 *M* NaCl, 50 m*M* $MgCl_2$, pH 9.5.

3. Methods

A novel cell line, designated HS-SYII, was derived from a synovial sarcoma of the monophastic fibrous type and harbors SYT-SSX1 transcripts ***(12)***. We used cultured H S-SYII cell clotting to establish the current protocol. Briefly, 1×10^7 cells are collected by centrifugation, fixed in formalin for 16 h at room temperature, and finally embedded in paraffin as surgically resected tissues. Prepared tissue sections were used as positive controls, whereas surgically resected other soft part tumor tissues were used as negative controls. We

bypassed the RT step to avoid the possibility of DIG-labeled probe hybridizing with SYT-SSX1 mRNA *in situ*. In our experimental conditions, we were unable to detect any significant signals without RT steps even in, H. S.-SYII cell clots. We believe that the amount of HS-SYII mRNA in paraffin-embedded synovial sarcoma tissues is insufficient for detection by simple *in situ* hybridization, or alternatively is degraded after the RT steps of the current protocol.

3.1. Preparation of Tissue Sections

1. Immediately after resection, the tissues are cut and fixed in 10% buffered formalin for 10 to 16 h at room temperature (*see* **Note 6)**.
2. Tissues are cut into 6- to 8-µm-thick sections (*see* **Note 7**). The sections are deparaffinized with lemosol and dehydrated with ethanol. For frozen tissues, the tissues are cut immediately, and then fixed in ethanol at room temperature for 5 min.
3. The sections are digested with 40 µg/mL proteinase K in, phosphate-buffered solution for 20 min at room temperature (*see* **Note 8**). Proteinase K is then inactivated by heating at 95°C for 5 min.

3.2. RT In Situ *Step*

1. Tissue sections are subsequently treated with RNase-free DNase at 37°C for 20 min (*see* **Note 9**). Inactivate DNase by incubating the specimens at 75°C for 10 min.
2. The RT procedure is conducted using reaction buffer. Fifty microliters of reaction buffer is added to tissue sections, sealed with Hybaid Easyseal, and incubated at 37°C for 10 min at 42°C for 30 min, 95°C for 5 min, and chilled on ice. The tissues are fixed with 80% ethanol at room temperature for 2 min (*see* **Note 10**).

3.3. PCR In Situ *Step*

1. After washing in washing solution, 50 µL of reaction mixture containing 5 U of AmpliTaq Gold polymerase (Perkin Elmer; *see* **Note 11**), 200 µ*M* of each deoxynucleotide triphosphate, and 50 n*M* of SYT-SSX primer (nested primers) is added to the glass slide and subsequently covered with an EasySeal cover. The final concentration of $MgCl_2$ is 4.5 µ*M* (*see* **Note 12**).
2. The PCR step is performed by heating at 95°C for 7 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Final elongation of PCR product is performed at 72°C for 20 min.
3. Tissues are incubated in PBS for 5 min and subsequently fixed in 100% ethanol for 5 min at room temperature (*see* **Note 13**).

3.4. Labeling of cDNA Probes and Hybridization Step

1. DIG easy hybridization buffer is added and the tissues are incubated at 55°C for 1 h.
2. SSX-SYT chimeric 200-bp cDNA was obtained from cultured HS-SYII with nested primers by using RNAzol and the RNA LA PCR kit.

3. Amplified SSX-SYT cDNA is labeled with DIG according to the random primed labeling procedure by using DIG high prime-labeling kits. Briefly, 1 µg of S SX-SYT cDNA in 16 µL of sterile water is heated to 95°C for 10 min and quickly chilled on ice. Four microliters of DIG high prime mixture is added to SSX-SYT cDNA and centrifuged briefly. After 16 h of incubation at 37°C, the mixture is heated at 95°C for 10 min and quickly chilled on ice.
4. The DIG-labeled cDNA is mixed with DIG easy hybridization buffer and then heated to 55°C.
5. Diluted DIG-labeled cDNA is added to tissues (*see* **Note 14**). The tissues are incubated at 55°C overnight (14–16 h).

3.5. Posthybridization Washes

1. Tissue slides are washed twice for 5 min with a solution containing 2X SSC and 0.1% SDS at room temperature.
2. The tissue slides are then washed twice for 15 min with a solution containing 0.1X SSC and 0.1% SDS at 55°C.

3.6. Immunohistochemical Detection of Labeled Probe

1. The tissue slides are washed with maleic acid buffer for 5 min at room temperature.
2. The tissues are incubated with blocking buffer for 30 min at room temperature.
3. The tissues are incubated with diluted anti-DIG-AP conjugate for 1 h at room temperature (*see* **Note 15**).
4. The tissue section is washed twice for 15 min with maleic acid buffer.
5. The tissue section is washed for 5 min with detection buffer.
6. The tissues are incubated with freshly prepared color solution at room tempera-hhhture (*see* **Note 16**).
7. Finally, the tissues are washed with phosphate-buffered saline. Representative examples are shown in **Fig. 4**.

4. Notes

1. Fixation of the tissues is the most important process for RT *in situ* PCR It is ideal to determine the fixing condition by using cultured cell clots in which the desired mRNA has been preliminarily identified.
2. Any glass slide can be used as an alternative to that described; however, glass slides in which tissues were not detached during any of the steps of the procedure and possess little or no contamination of RNase should be preferentially selected.
3. RT, buffer, dNTPs, and RNase inhibitors are included in this kit.
4. Martinez et al. ***(4)*** reviewed a few models of thermocyclers for *in situ* PCR and selected this OmniSlide system based on ease and flexibility of operation.
5. This kit contains specific antibody to DIG 10X blocking solution, and color-substrate solution.
6. We sometimes encountered difficulties in detecting signals by RT *in situ* PCR when fixing large tissues (more than 1 cm^3) without adequate precutting. These difficulties may be caused by degradation of mRNA before fixation. Most of the

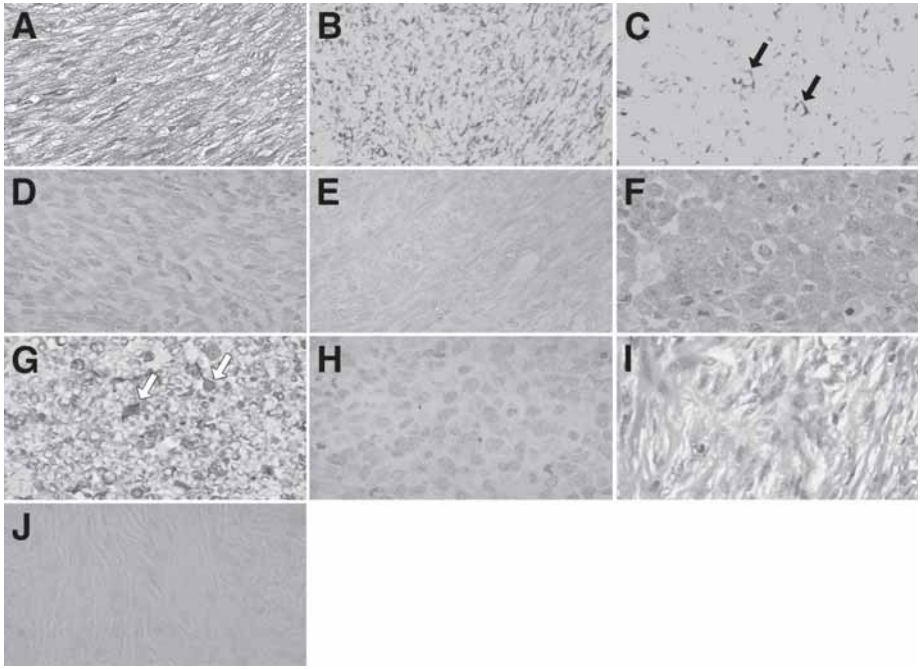

Fig. 4. Representative results of RT *in situ* PCR. A synovial sarcoma, in which SYT-SSX chimeric gene was detected by standard RT-PCR in tube, was stained according to the current protocol. Spindle tumor cells (**A**, hematoxylin and eoxin staining, original magnification, ×400) were stained by RT *in situ* PCR (**B**, ×200; **C**, ×400). Note the positive signals in cytoplasm (**C**, arrow). As a negative control, the RT step was bypassed (**D**, ×400). Omission of the primers in the PCR mixtures resulted in elimination of the signals (**E**, ×400). In **D** and **E**, nuclei were stained with hematoxilin after RT *in situ* PCR to identify the cells. Cell clot of HS-SY II also was stained with current protocol (**G**, ×400, white arrows indicate cytoplasmic staining). Omission of the primers again eliminated the signals (**H**, ×400, hematoxylin staining was added). Spindle cell tumor of Schwannoma (**I**, hematoxylin and eosin staining, ×400) was not stained by, RT *in situ* PCR (**J**).

suitable fixed samples in our experiments were those where the tissues were immediately frozen in liquid nitrogen and cut into 6- to 8-μm thick sections and subsequently fixed in ethanol for 5 min at room temperature. Alternatively, tissues may be fixed in 10% buffered formalin or 1–4% paraformaldehyde for 12 to 16 h or 20 min, respectively, at room temperature.

7. Special care should be taken to avoid RNase contamination during tissue preparation. This applies to all reagents and equipment, such as cutting knives, which should be pretreated to inhibit RNase activity or acquired new from the manufacturer.
8. In order to gain sufficient signal intensity, the tissues are cut into 6- to 8-μm thick sections. The permeabilization step facilitates the entry of reagents into the tis-

sues. There is no standard permeabilization method, including the selection of proteinase. However, we use proteinase K because we expect that it also directed to RNase in the tissue section.

9. We may omit this step during preliminary experiments in cases where we do not need to discriminate between the localization of RT *in situ* PCR signals. When sufficient signal intensity is detected in the cytoplasm, this step may be added to remove any signal because of amplification arising from genomic DNA.
10. In many protocols, extensive washing with various concentrations of SSC is performed after the RT reaction. We did not find any benefit in this wash step because diffusion of cDNA out of the tissue could result in poor PCR efficiency. Instead of extensive washing, we found that incubation of the tissue section in 80% ethanol was able to fix the cDNA *in situ* and wash the buffer out of the tissues.
11. We used hot-start *Taq* polymerase to reduce nonspecific binding of primers to the cDNA template.
12. It generally is accepted that the efficiency of *in situ* PCR is less than standard PCR performed in a tube. To achieve sufficient amplification, it is important to increase the final concentrations of *Taq* polymerase, $MgCl_2$, and/or primers.
13. This postamplification treatment also may remove a part of products on the outside of the cells. Amplified products formed during PCR may readily diffuse from the site in which the original cDNA template was located. We speculate that the poor efficiency of RT *in situ* PCR may be caused by this phenomenon, also known as the diffusion artifact Once the products have floated into the reaction buffer, it can be a template for subsequent PCR amplification, thereby seriously decreasing the intensity of the final signal at the original site. Overlaying tissue sections with a thin layer of agarose may help to decrease the diffusion artifact, although we were unable to detect any significant benefit in our experiments.
14. Dilution rates are dependent on individual experimental conditions. However, we usually diluted labeled cDNA probe 20-fold with DIG easy hybridization buffer.
15. We usually dilute the antibody 1000-fold. However, the dilution rate should be optimized for individual experimental conditions.
16. During color development, do not shake the tissues. Exposure to light should be restricted as much as possible. The color development is usually performed by overnight incubation but can be shortened by 2 to 3 h when sufficient signal intensity is observed.

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18

In Situ Reverse Transcription PCR on Plant Tissues

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Summary

In situ detection techniques allow specific nucleic acid sequences to be exposed in morphologically preserved tissue sections. In combination with immunocytochemistry, *in situ* detection can relate microscopic topological information to gene activity at the transcript or protein levels in specific tissues. The advantage of *in situ* methods over the conventional techniques (e.g., Northern blot, reverse transcription polymerase chain reaction [RT-PCR], or real-time PCR) is that they allow the investigatation of the putative spatial distribution of nucleic acid products activity in a heterogeneous cell population. In this chapter, we describe a protocol for *in situ* RT-PCR detection of specific messenger RNA in cucumber (*Cucumis sativus*), although this protocol can be used for any plant species, floral buds, and somatic embryo tissue sections on glass microscope slides. A successful *in situ* RT-PCR procedure requires the optimization of many conditions related to the tissue types used, for example, a cell's age, size, and composition, which may influence the detection of RT-PCR products, as well as specific transcript availability. Moreover, parameters, such as the fixation time, thermal cycling set-up, and the time of detection of RT-PCR products, also should be optimized. The importance of the other factors also is estimated in the protocol. In addition several types of controls that are necessary for a trustworthy *in situ* RT-PCR method are being discussed.

Key Words: Direct *in situ* RT-PCR; *Cucumis sativus*; floral buds; somatic embryos; paraffin tissue sections; digoxigenin-11-dUTP; alkaline phosphatase substrate.

1. Introduction

There are several common names for *in situ* polymerase chain reaction (PCR), which is performed based on RNA. Nuovo ***(1)***, who was one of the first developers of the method, called it reverse transcription (RT) *in situ* PCR, but since then, other names have been created for it, such as *in situ* complementary (c)DNA***(2)*** or *in situ* reverse transcriptase PCR ***(3)***, which are present in literature to date ***(4)***.

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In situ RT-PCR combines the sensitiveness of PCR amplification with spatial localization of products to monitor the appearance of specific transcripts in the tissue sections. Therefore, *in situ* RT-PCR defines a powerful tool for the low-abundance transcript detection ***(5)*** because the revealing threshold can be as low as one or two copies per cell. In comparison, *in situ* hybridization detects 10 to 20 copies per cell ***(6)***. This method has not been used to a large extent in plants, but it provides several advantages over the classic *in situ* RNA hybridization, which was widely discussed recently ***(7)***.

The first application of *in situ* RT-PCR for the plant tissue was reported by Woo et al. ***(8)*** and described the expression of the *HIS 3;2* gene (encoding the H1 histone) in the single detached border cells of pea seedlings. The subsequent papers on applying *in situ* RT-PCR technique to the plant material regarded several different tissues and genes ***(9–16)***.

In situ RT-PCR techniques can be classified into two groups based on labeling and detection systems used (**Fig. 1**). During the direct *in situ* RT-PCR, digoxigenin (biotin or fluorescein)-labeled nucleotides ***(13,14)*** or primers ***(15)*** are incorporated into the PCR product, leading to a direct signal detection. To the contrary, the indirect signal detection for *in situ* RT-PCR occurs when the PCR product is subsequently visualized by hybridization with specifically labeled probe ***(16)***. The direct *in situ* RT-PCR can be a quicker alternative to the indirect technique because it avoids the subsequent *in situ* hybridization step.

Although RT-PCR technique generally is adjusted by optimizing RT-PCR mixture and timing, for the successful procedure it also is crucial to set up optimal conditions for each new tissue in respect of its cells size and composition (e.g., lignified walls), as discussed previously ***(7)***. Engler et al. ***(17)*** described an *in situ* protocol suitable for obtaining the optimal results for different *Arabidopsis* tissues. Lee and Tegeder ***(7)*** observed that the thickness of the tissue sections and proteinase K pretreatment strongly increased the probability of successful application of *in situ* RT-PCR. Furthermore, the fixation and embedding processes also have a noticeable influence on the *in situ* RT-PCR results ***(14)***.

This chapter is aimed at providing a laboratory protocol for the *in situ* RT-PCR optimized for localization of transcripts in the cucumber floral buds and cucumber somatic embryo tissue sections, which also can be used for other plant species. Additionally, we provide a comprehensive procedure of *in situ* RT-PCR while discussing the main steps of this technique and providing a detailed list of materials effectively used in our laboratory for it.

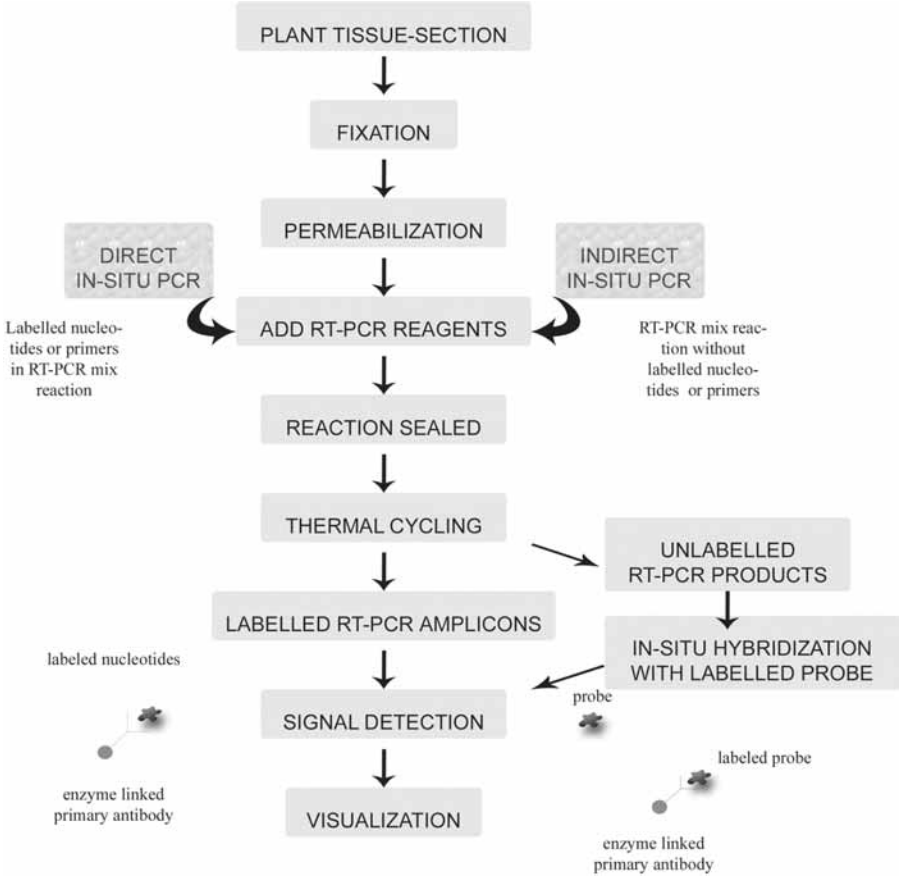

Fig. 1. Schema of *in situ* RT-PCR. Direct *in situ* RT-PCR-labeled nucleotide or primer is incorporated in RT-PCR mix reaction. Indirect *in situ* RT-PCR-labeled probe is incorporated during a hybridization step.

2. Materials

2.1. Tissue Preparation

As with all RNA work, precautions against RNase contamination should be taken. All solutions were prepared using 0/1% diethylpyrocarbonate-(DEPC) treated MilliQ H_2O. Always use gloves. Metallic tools, the glass dish, and cover are individually wrapped in aluminum foil and sterilized overnight at 180°C.

The majority of the chemicals are from Sigma-Aldrich Chemie in Steinheim, Germany. Chemicals from other company are noted.

1. To produce comparable material, all plants should be grown in tightly controlled environmental and developmental conditions. For cucumber (*Cucumus sativus*) plants are cultivated in a greenhouse under the same light regime with a minimum of 300 to 400 μ*M* photons m^{-2} s^{-1} at 20°C (night) and at 25°C (day) with a 8- to 16-h photoperiod. Healthy-looking, similar-size buds of the same shape and color are selected for experimental purposes. For each repetition, a pool of cucumber tissue should be taken. Embryos are collected with embryogenic suspension culture in phases: 0, 6, and 24 h and 3, 7, and 14 d after induction (after removing 2,4-dichlorophenoxyacetic acid).
2. Absolute ethanol (99.8% (Polmos, Kutno, Poland).
3. Formaldehyde 37% solution (12.3 *M*). Hazardous; store in chemical box at room temperature.
4. Acetic acid; store in chemical box at room temperature.
5. FAA fixative: 2% formaldehyde, 5% acetic acid, 60% ethanol. Prepare immediately before use and store on ice.

2.2. Tissue Embedding

1. DEPC. Very toxic. Stock store at 4°C.
2. RNase-free water (DEPC water): to MilliQ H_2O add DEPC to 0.1%, shake well, leave at 37°C overnight, then autoclave twice for 40 min to destroy excess the DEPC, and store at room temperature.
3. Ethanol solutions: 70%, 85%, 95%, and 99.8%.
4. HistoClear (Histochoice Clearing Agent): irritant; store in chemical box at room temperature.
5. HistoClear: ethanol ratio of 2:1; prepare immediately before use and store under exhaust at room temperature.
6. HistoClear: ethanol ratio of 1:2; prepare immediately before use and store under exhaust at room temperature.
7. Embedding medium: Paraplast Plus; store at room temperature, but melt before use to 58–60°C.

2.3. Microscope Slide Sectioning

There is no need for a special slides preparation in case of SuperFrost Plus glass slides being used. *Cucumis* embryo and flower bud paraffin sections perfectly attach to these slides, and Superfrost Plus slides optimize *in situ* methods. If you do not have SuperFrost glasses, you should prepare clean, RNase-free glass slides. To coat the slide for tissues adhesion, dip them in 2% 5-aminopropyltrithoxysilane (TESPA) in dry acetone for 5 to 10 s. Quickly rinse them (twice in acetone and once in distilled H_2O), then air-dry. Slides coated with TESPA can be kept for several months.

1. "Ready-to-use" glass slides (SuperFrost Plus, Menzel-Glaser, Germany), 25 × 75 × 1 mm.

2. MiliQ-water.
3. Embedding tissue.

2.4. Pretreatment of Slides

1. HistoClear.
2. Ethanol solutions: 99.8%, 95%, 85%, 70%, 50%, and 30%.
3. 0.02 *M* HCl: add 1.2 mL of concentrated HCl to 60 mL of water.
4. 20X standard saline citrate (SSC): sodium citrate solution: 3 *M* sodium chloride, 0.3 *M* Tri-sodium citrate. To prepare 20X SSC, add 175.3 g of NaCl and 88.2 g of sodium citrate adjust to pH 7.0 and add up to 1 L of DEPC-treated water, autoclave, stored 2 to 3 wk at 4°C.
5. 0.5 *M* Ethylene diamine tetraacetic acid (EDTA) pH 8.0: add 18.61 g of EDTA to 80 mL of DEPC-treated water, to prepare 100 mL solution; stir with a magnetic stirrer, dissolve by adjusting to pH 8.0 with NaOH (10 *M*), and complete volume to 100 mL, then autoclave 17 min at 121°C.
6. Pectinase from mold: (Fluka Chemika, Buchs, Switzerland), store at 4°C.
7. Pectinase buffer: 0.1 *M* sodium acetate, 5 m*M* EDTA. Per 50 mL, add 0.41 g of sodium acetate, 0.09 g of EDTA, and up to 50 mL of water. Adjust to pH 4.5, store at 4°C.
8. 10% Pectinase solution: put 1.5 mL of 10% pectinase buffer and add 15 mg of pectinase into Eppendorf tube. Mix these compounds together gently for a minimum of 4 h and store at 4°C.
9. Proteinase K. Store at 4°C.
10. Proteinase K buffer: 100 m*M* Tris-HCl, 50 m*M* EDTA, pH 8.0. Add 3.025 g of Tris-HCl, 4.652 g of EDTA, and up to 250 mL of DEPC water. Adjust to pH 8.0 and autoclave; store at –20°C.
11. 10% Glycine solution: add 25 g of glycine and up to 250 mL of DEPC water, autoclave, and store at 4°C.
12. 10X phosphate-buffered saline (PBS): adjust to pH 7.4 and autoclave. Keep at 4°C for 2 to 3 wk.
13. DNase buffer: 0.1 *M* sodium acetate, 5 m*M* $MgSO_4$, pH 5.5. Add 0.41 g of sodium acetate and 0.0615 g $MgSO_4 \cdot 7H_2O$ and up to 50 mL DEPC water. Autoclave and store at –20°C.
14. RNase-free Dnase I (Roche Molecular Biochemicals, Mannheim, Germany). Store at –20°C.

2.5. In-the-Tube RT-PCR

1. Pair of primers: designed by the user (*see* **Note 1**), of known concentration (can be stable at –20°C).
2. Gene Amp r*Tth* DNA Polymerase and EZ Buffer Pack: (EZ rTth kit, PE Biosystems, Nortwalk, CT) all reagents should be stored at –20°C. The kit includes r*Tth* DNA polymerase, 5X EZ Buffer, and 25 m*M* $Mn(OAc)_2$. r*Tth* polymerase is a dual-activity enzyme, that is, a reverse and high-temperature *Taq* polymerase. It makes the one-step RT-PCR reaction possible.

3. 10 m*M* each of dNTPs (Roche Molecular Biochemicals). Store at –20°C. Mix four dNTPs into Eppendorf tubes. Store in aliquots to avoid constant freezing and unfreezing.
4. Bovine serum albumin (20 mg/mL; Fermentas, Vilnius, Lithuania). Store at –20°C.

2.6. RT-PCR Step of In Situ RT-PCR

To the amplifying solution described in **Subheading 2.5.**, add digoxigenin-11-dUTP (25 n*M*; Roche Molecular Biochemicals) and store at –20°C.

2.7. Detection System

1. Prepare 2X, 1X, and 0.5X SSC solutions.
2. Maleic buffer: 100 m*M* maleic acid, 150 m*M* sodium chloride. Add 11.61 g of maleic acid and 8.76 g of NaCl, adjust to pH 7.5, autoclave, and store at 4°C.
3. Triton X-100. Store at room temperature.
4. Goat serum: (Vector Laboratories, Burlingame, CA). Store at 4°C.
5. Maleic-serum-Triton solution: 1 mL of maleic buffer, 40 µL of goat serum, and 3 µL of TritonX-100.
6. For digoxigenin system: use the antidigoxigenin–alkaline phosphatase (AP) *Fab* fragments (Roche Molecular Biochemicals) 150 U/200 µL. Store at 4°C.
7. Maleic-serum-Triton-AP solution: 500 µL of maleic buffer–serum–Triton solution and 0.5 µL of antidigoxigenin–AP *Fab* fragments.
8. 4-Nitroblue tetrazolium chloride (NBT; Roche Molecular Biochemicals). NBT is very toxic. Store at –20°C in the dark.
9. 5-Bromo-4-chloro-3-indolylphosphate-4-toluidine (BCIP; Roche Molecular Biochemicals). BCIP is very toxic. Store at –20°C in the dark.
10. Magnesium buffer: 100 m*M* Tris-HCl, 100 m*M* NaCl, 50 m*M* $MgCl_2$. Add 5 mL of 1 *M* Tris-HCl, pH 8.0; 0.29 g of NaCl, 2.5 mL of $MgCl_2$, and up to 50 mL of sterile water. Adjust to pH 9.5.
11. Prepare magnesium–NBT-BCIP solution: 500 µL of magnesium buffer, 1.75 µL of BCIP, and 2.25 µL of NBT.

2.8. Other Equipment

1. The frame and the cover: (65 µL of EasySeal Hybaid Limited, Middlesex, UK); the size of the frame will depend on the size of the tissue.
2. Microscope (Olympus BX 60) with image analysis system (AnalySIS Soft Image System GmbH, Münster, Köln, Germany) and video camera (CCD-IRIS/RGB Sony Image System GmbH, Munster).
3. Slide storage boxes: sealable (Kartell, Italy). Boxes that hold 25 and 100 slides are useful.
4. Pipets used for RNA only.
5. Air vacuum (Cole-Parmer, Chicago, IL).
6. Thermalcycler with *in situ* block: (Biometra, Annalytik GmbH, Göttingen, Germany).

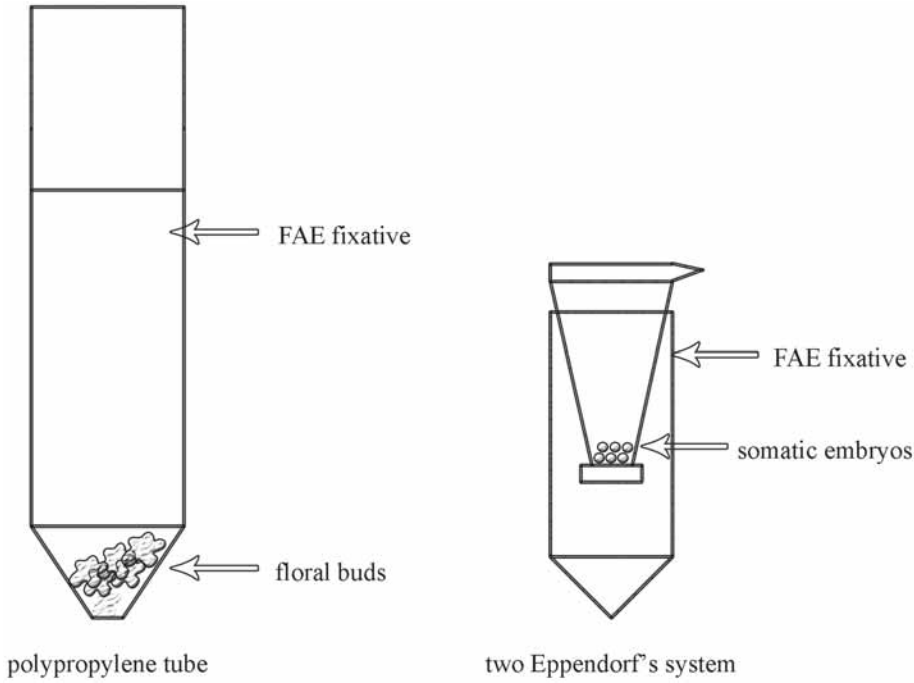

Fig. 2. Variations of possible placement manner for the fixation of cucumber floral buds and cucumber somatic embryos.

7. Microtome (Microm Laborgerate GmbH, Walldorf, Germany).
8. Humidified box: a small plastic box with a sterile, damp gauze at the bottom; this box can be used for holding slides during incubation with different reagents using in the reaction.

3. Methods

3.1. Tissue Preparation

1. Immediately after dissecting the plant material, throw the tissue into a fresh, cold FAA fixative and store on ice (*see* **Note 2**). Do not fix more than 1 cm^3 of tissue per 40 mL of fixing solution. Cucumber floral buds are put in polyester tubes and somatic embryos into a "two-Eppendorf system" (**Fig. 2**). A 1.5-mL Eppendorf tube is filled with the fixative solution and contains another 0.5-mL Eppendorf without a bottom, which is mounted with sticky nylon net (Ø stitch: 20 µm). The embryos are then laid on the net with small stitches, which helps collect tissues after fixation and transfer to the next solution.
2. Tubers with tissues in fixation solution should be transferred to the vacuum oven. Use a vacuum to degas the plant tissues for cucumber buds for approx 3 min and

for embryos for approx 1 to 2 min, which can improve penetration of tissues by fixative. After degassing, the fixed tissue should be sunk on the button of tubes and then stored at 4°C overnight (*see* **Note 3**).

3.2. Tissue Embedding

It is necessary to use at least 10 times greater volume of the solvent of that of the prepared tissue.

1. Remove the fixative solution and replace it with 70% ethanol. Use a vacuum for a better infiltration and store for approx 6 h at 4°C. Remove 70% ethanol by replacing it with a fresh 70% ethanol and then degas tissue samples in vacuum oven and store at 4°C overnight.
2. Dehydrate the fixed samples by incubating them in gradient series of ethanol solutions in H_2O (85%, 95%, followed by two cycles in 99.8% ethanol), each stage for 1 h at room temperature under the degas system.
3. Incubate tissues with 1 volume of HistoClear in 2 volumes of ethanol, employ a vacuum, and leave at room temperature for 1 h, then repeat this step with 2 volumes of fresh HistoClear in 1 volume of ethanol mixture.
4. Transfer the plant samples to 100% HistoClear, employ a vacuum, and leave at room temperature for 1 h. Repeat the 100% HistoClear incubation for 1 h for a total ethanol replacement.
5, The Paraplast Plus should be melted at 58 to 60°C before embedding tissues in a vacuum oven (*see* **Note 4**). After removing the plant samples from HistoClear, place them into glass bakers with melted Paraplast in the vacuum oven at 60°C overnight. Replace with freshly melted Paraplast twice a day for 3 to 4 d to obtain a perfect infiltration, then for the last step, the samples should be transferred into an aluminum mold or small Petri dishes. Finally degas in the vacuum oven overnight. On the next day, arrange the tissues position using a bacteria inoculation needle and then immediately put the embedded materials on ice (partially thawed with ethanol and water) for quick cooling. The paraffin blocks can be stored at room temperature or at 4°C indefinitely.

3.3. Microscope Slide Sectioning

For our system, we used glass slides (SuperFrost Plus), which can be used directly without the need for any special adhesives or protein coating.

1. Using a sharp single-edged razor blade trim the excess wax from the edges of the embedded tissue block, get a tissue sample of the pyramid shaped structure, and place it onto microtome block.
2. Cut a 7-µm thin ribbon section from the block. We have applied this thickness to both floral buds and cucumber embryos to obtain optimal results.
3. Sections submerged into warm water in microtome are ready for careful adherence onto the surface of the slides (*see* **Note 5**). Slides should be dipped into the water and sections caught onto the surface of the slides. Remove the excess water

from the tissue, by rattling gently on sterile scraps of paper or using the corner of a paper sheet.
4. Place the slides on a heat tray at 40–42°C overnight (*see* **Note 6**). Then, transfer them to the slide storage boxes located in a dry place for a long storage.

3.4. Pretreatment of Slides

We recommend that the following pretreatments should take place in laminar flow cabinets (to protect against dust and especially RNase contamination) at room temperature. Incubate paraffin sections in HistoClear to remove paraffin for 10 min, and then repeat this step in new HistoClear.

1. Immerse slides in 100% ethanol for 1 min, then repeat this step with a fresh 100% ethanol.
2. Rehydrate the mounted tissue sections by washing the slides in each of a graded series of solutions of ethanol in DEPC water by ethanol series (95%, 85%, 70%, 50%, and 30%) and finally, transfer to the DEPC-water. At each step, wash the slide by repeatedly dipping it for approx 1 min.
3. Incubate the slides in 0.02 *M* HCl for 20 min (*see* **Note 7**).
4. Transfer the slides into 2X SSC for 30 min. Remove excess 2X SSC from the slide surface by gently shaking down on sterile sheets of paper. Place each slide into the plastic humidified box, on the blotting paper moisten with DEPC water.
5. Firmly closing the humidified box lid is crucial (*see* **Note 8** and **Fig. 3**).
6. For each slide, add approx 70 to 100 µL of pectinase buffer to cover completely tissue section and incubate the slides for 10 min.
7. Remove the pectinase buffer from slides by shaking down on a sterile blotting paper.
8. Apply approx 50 µL of 1% pectinase in pectinase buffer and incubate for 10 min (*see* **Note 9**).
9. Remove the remains of pectinase (1%) from slides by shaking down on sterile blocking papers.
10. Apply approx 100 µL of proteinase K (2 µg/mL final concentration) in proteinase buffer for each slide (to cover the tissue section completely) and quickly transfer to 37°C, then incubate for exactly 5 min in a humidified box (*see* **Note 10**).
11. Wash slides in glycine (2%) in 1X PBS for 1 min. Wash another two times in 1X PBS for 5 min. Remove the excess of 1X PBS from the slide surface by gently shaking down on sterile blotting paper.
12. Put each slide for a second time into the humidified box and apply 150 µL of DNase buffer (tissue sections should be completely covered). After short incubation replace with a new DNase buffer and subsequently incubate for 45 min.
13. Remove DNase buffer by shaking down on a sterile paper towels.
14. Apply 8 U RNase-free DNase I in 50 µL of DNase buffer onto each section.
15. Tissues should be treated in a humidified box at 37°C overnight (*see* **Note 11**).
16. The digestion of tissues with DNase is recommended to avoid amplification of nuclear DNA.

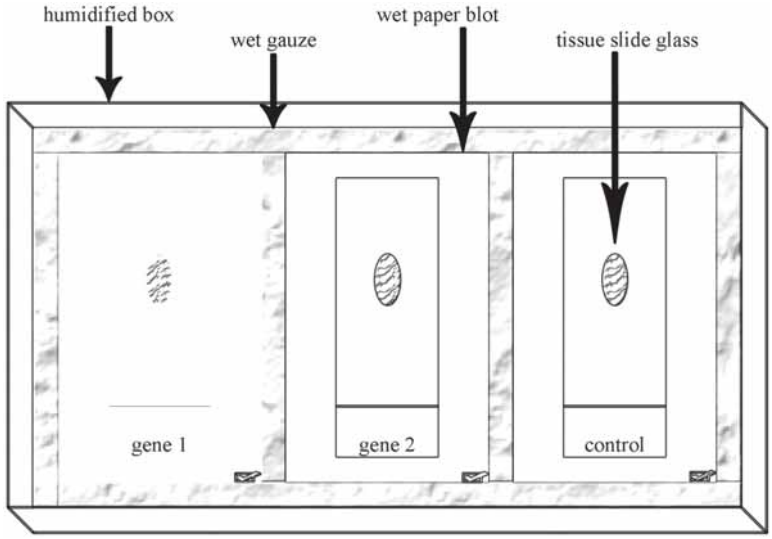

Fig. 3. Humidified box used for digestion steps during *in situ* RT-PCR.

17. The next day, wash the slides in the following solutions: 0.5 *M* EDTA, 2X SSC, 1X SSC, 0.5X SSC, and DEPC water for 10 min each.

3.5. In-the-Tube RT-PCR

Control specimens without r*Tth* polymerase and with or without primers always should be included. Use also an amplification mixture from under cover slip as control. (*See* **Note 12**).

One of many controls deployed is the "in-tube RT-PCR reaction," which can be assumed as performed, if the detected transcripts are present in the tissue.

1. RNA from selected tissues should be extracted using convenient protocol.
2. Reaction mix contains: 5U r*Tth* Polymerase, 5X EZ buffer, 2.5 m*M* $Mn(OAc)_2$ 0.3 m*M* dNTPs, 0.064% bovine serum albumin, 1.2 µ*M* of each forward and reverse primers, and 1 µg of RNA.
3. Cycling reaction set-up: 61°C for 30 min; 94°C for 3 min; 30 cycles of two-temperature PCR (94°C for 75 s, 61°C for 75 s); and the final extension in 61°C for 7 min. PCR is completed in an UNO II Thermalcycler.
4. The RT-PCR products were visualized by gel electrophoresis on 2% agarose gel with EtBr.

3.6. RT-PCR Step of In Situ *RT-PCR*

Parallel to washing (*see* **Subheading 3.4.**, **step 15**), the RT-PCR reaction mixture needs to be prepared. Necessary reagents are stored at –20°C and first have to be gently thawed on ice.

1. The reaction mixture should be prepared in Eppendorf tube. The mixture contains: 5X EZ buffer, 0.3 m*M* dNTPs, 2.5 m*M* $Mn(OAc)_2$, 5u r*Tth* Polymerase, 0.064 bovine serum albumin, 25 m*M* digoxigenin 11-duTP, and 1.2 m*M* each of primers in a total reaction volume of 50 µL.
2. Transfer the slides from water incubation onto a sterile blotting paper.
3. When the part of the glass slide around tissue sections is dry, mount EasiSeal frame and stick it tight to the glass (do not touch the tissue sections, which should be inside the frame). Remove the paper protecting the frame.
4. Transfer the slides into the plate heated up to 61°C. This step needs to be processed promptly and without pause.
5. Apply RT-PCR reaction mixture onto the frame, starting from one edge of the frame.
6. Gently and slowly stick a flexible plastic cover slip (EasiSeal) to the frame from the edge where the reaction mix was applied (*see* **Note 13** and **Fig. 4**).
7. Transfer slides to the *in situ* thermocycler block preheated to 61°C. Cycling reaction set-up: 61°C for 30 min; 94°C for 3 min; 32 cycles of two-temperature PCR (94°C for 90s, 61°C for 90s); and the final extension in 61°C for 7 min. The optimal lid temperature for cucumber floral bud sections is 105°C and 70°C for cucumber somatic embryos. PCR is completed in an UNO II Thermalcycler with *in situ* block. Several controls can be applied to this step (*see* **Note 14**). The reaction without primers is the most informative (for that, H_2O is added instead of primers).

Optimization of *in situ* RT-PCR requires many different experimental controls to ensure that no false-negative or false-positive results are obtained. After optimization, we routinely use only one control (reaction with water instead of primers), which in our opinion is capable of identifying any unspecific amplification. These controls served to confirm that the signal detected was derived from the amplified mRNA in the sections (**Fig. 5**).

3.7. Detection System

All detection steps are being performed at room temperature with freshly prepared buffers.

1. After the amplification RT-PCR, frames with covers are gently removed from each microscope slide.
2. Wash the slides twice in 2X SSC for 5 min. Rinse the slides in the following solution: 1X SSC and 0.5X SSC.
3. Remove remaining drops of 0.5X SSC buffer by shaking down on sterile blotting paper (by gently hitting the paper towel with vertically positioned glass slide) and put each slide in humidity chamber.
4. Quickly apply 300 µL of maleic buffer into the slide (sections should be covered with liquid), then remove its excess by shaking down on a sterile blotting paper.
5. Repeat the maleic buffer treatment with 100 to 200 µL (sections should be covered with liquid) for 10 min.
6. Remove maleic buffer by shaking down on a sterile blotting paper.

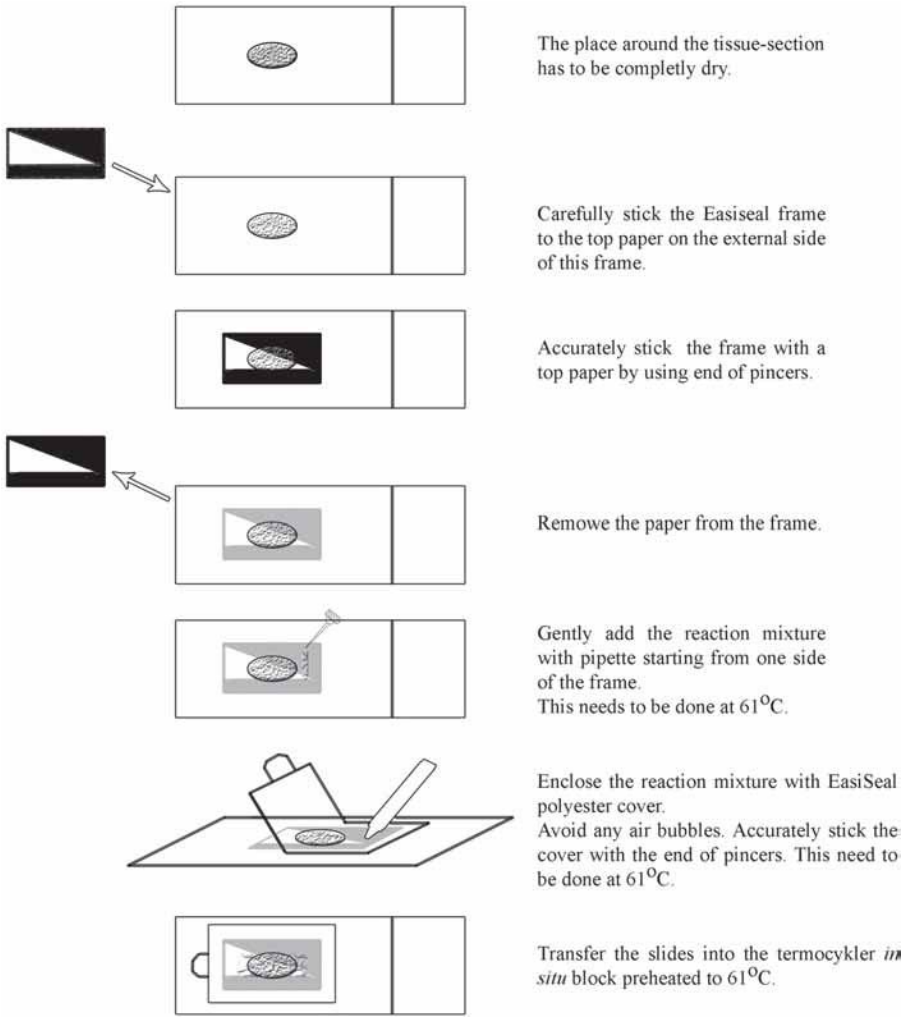

Fig. 4. The placement of RT-PCR reaction mixture on the tissue section.

7. Add 150 μL of maleic buffer–serum–Triton solution onto tissue, and incubate for 30 min.
8. Remove maleic buffer–serum–Triton solution by shaking down on sterile blotting paper.
9. Into fresh maleic buffer–serum–Triton solution, add antidigoxigenin antibody, conjugated with AP diluted to 1:1000 (maleic buffer–serum–Triton–AP solution), and incubate tissue for 2 h.
10. Remove a maleic buffer–serum–Triton–AP solution by shaking down on a sterile blotting paper.

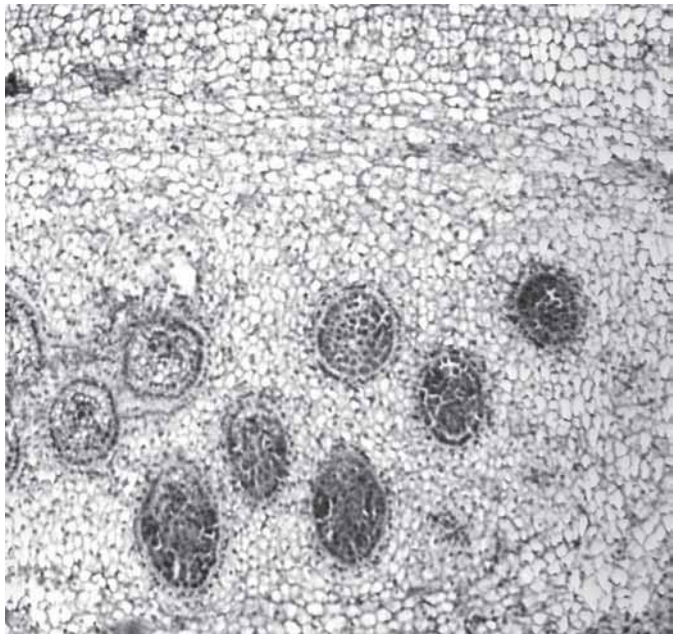

Fig. 5. Example of *in situ* RT-PCR control reaction without DNase pretreatment in cross-sectioned female bud of cucumber (*Cucumis sativus* L.). Signals were detected in every cell nucleus. Magnification ×100.

11. Wash the slides in magnesium buffer.
12. Remove the excess of the magnesium buffer by shaking down on a sterile blotting paper.
13. Repeat washing in magnesium buffer for 10 min. The unbound antibody conjugates will be removed this way.
14. Remove the magnesium buffer by shaking down on a sterile blotting paper and transfer slides into the humidified box.
15. Develop signal depicted by the color-substrate 0.3 mg/mL NBT and 0.2 mg/mL BCIP substrates. In the darkroom, gently apply (according to the supplier's recommendations) freshly prepared color-substrate on the tissue sections (slides need to be still in a humidified box, on the paper towels soaked in sterile water).
16. Monitor the color reaction development (blue-purple staining) on the tissue sections under microscope every few minutes. The color was developed for approx 15 min for cucumber embryos and 8 to 10 min for cucumber floral buds (*see* **Note 15**).
17. The color reaction can be stopped by incubation in water. Leave the slides in sterile water at 4°C for 12 h (color intensifies overnight in a fridge).
18. After washing in H_2O, the slides are air-dried and mounted in balsam oil (*see* **Note 16**), then covered with a cover slip. The drying time is approx 12 h.

19. For documentation-obtained results it is recommended that one use a microscope with the phase-contrast lenses and dark field. Try the lenses in the range of ×20 to ×100. Sections were observed and documented with a CCC-IRIS/RGB. Additionally, we have used the analySIS system connected to the Olympus light microscope to visualize the results after *in situ* RT-PCR procedure on the cucumber tissue-sections (**Fig. 6**).

4. Notes

1. The primers have to be designed to amplify a short fragment of complementary DNA not exceeding 250 base pairs. Larger fragments may be not completely reverse transcribed because of their secondary structure, and the nucleic acid can be partially degraded. To check how correctly the primers were designed, we performed the control reaction, with cucumber RNA as a target, in a tube.
2. Several fixatives can be used successfully for the preservation of plant tissues, for example, formaldehyde, glutaraldehyde, and a mixture of ethanol and acetic acid, as we described previously ***(14)***. Time of fixation should be optimized for each individual object, as it is one of the crucial factors. Most often the proper fixation time is 24 to 48 h. The fixation time conditions are very strictly dependent on protease digestion, and any change of the fixation protocol determines necessary changes in protease digestion circumstances. Insufficient digestion makes it difficult for the reagents to access the nucleic acids. However, overdigestion may cause outflow of the amplification products from the cells, when the reaction takes place and often leads to a loss of tissue architecture. Thus, any changes in fixation method should be correlated with optimization of protease digestion.
3. As mentioned previously, the volume of fixative depends on the amount of tissue being fixed. After applying vacuum gently, release it slowly as to facilitate the access of fixative into the tissue. Pieces of tissue that are well infiltrated with fixative should sink to the bottom of the tube as liquid replaces air in the tissue.
4. The manufacturer of Paraplast Plus warns against overheating. The temperatures higher than 62°C may adversely affect sectioning of the embedded tissues.
5. Heat the DEPC-water in microtome to release the paraffin sections. Sectioning process should be smooth and slides easy to observe under microscope. Transfer the ribbon into the water with a small paintbrush, and then allow it to float for 2 to 3 min and pick up the section with a microscope slide.
6. We found that treatment of slides at 42°C was important for optimal tissue adhesion to the slides (overnight). Slides were in a closed thermal box to prevent contamination.
7. The efficacy of the HCl is unknown.
8. For the incubation steps, we used closed plastic boxes containing gaze and moistened paper towels, which provided the necessary humidified conditions for several incubation steps (**Fig. 3**).
9. The pectinase treatment of the tissue prevents from an unspecific cell walls staining during the detection step.

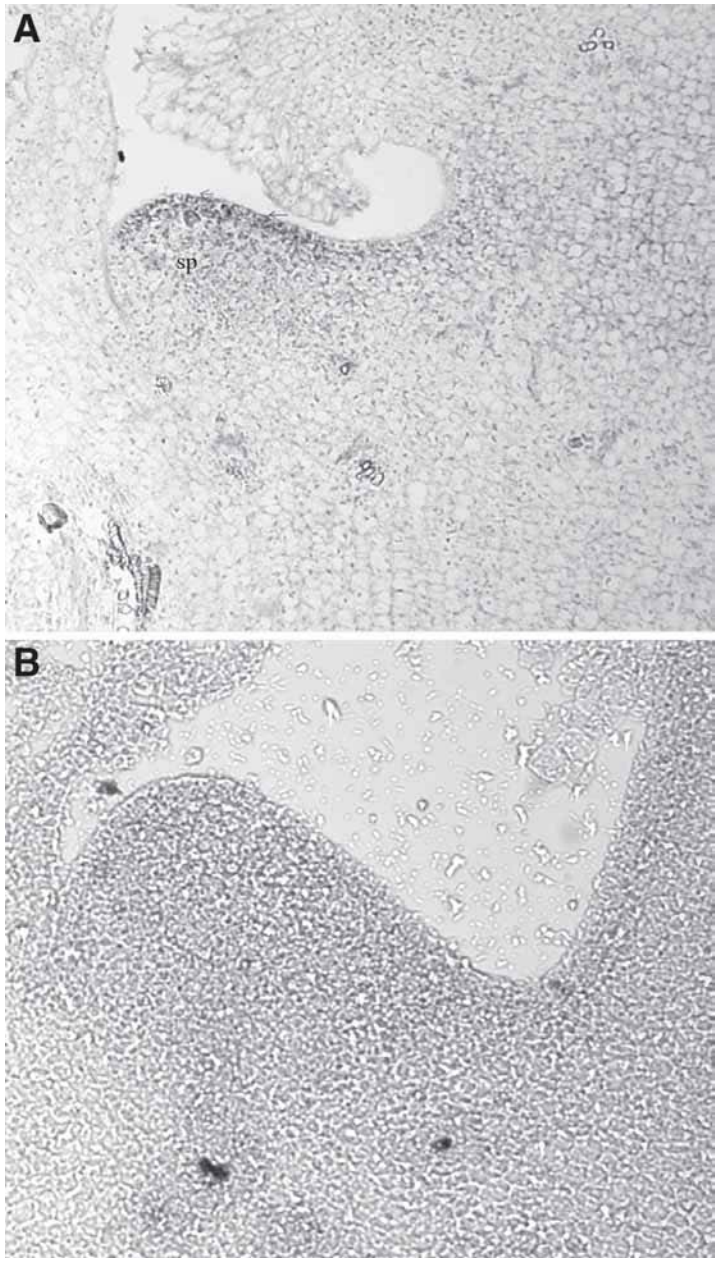

Fig. 6. *In situ* RT-PCR in cross-sectioned female bud of cucumber (*Cucumis sativus* L.). (**A**) reaction with specific 157B10 primers. Localization signals of transcripts in stamen primordium (sp). (**B**) Negative control reaction without specific primers in the RT-PCR mix. Magnification ×100.

10. The optimal time of proteinase K digestion was tested twice for the fixation of floral buds (for 48 and 24 h, respectively). The buds fixed for 48 h were treated with proteinase K for 15, 30, 45, and 60 min. The best results were achieved after 45 min of the proteinase K digestion. The morphology of the tissue was well retained, and interpretation of results was unambiguous. The signal was recognized as a purple-black dye at the site of the label. The 30- and 15-min durations turned out to be too short and led to lack of signal. The extension of the reaction time up to 60 min produced morphological distortions to the point that interpretation of results became impossible, so that unspecific signals appeared in every cell. We applied the same four time intervals of proteinase K digestions to the buds that were fixed for 24 h. It turned out that all tested times were too long. After signal development, only the unspecific signals were observed and the tissue morphology was poorly preserved. Three new times of digestion were tested (5, 15, and 20 min). Negative control without the proteinase K digestion also was prepared. The shortest time (5 min) proved to be optimal. The tissue was well preserved, and the signal was clear and specific. The reaction without proteinase K treatment was carried out, but no signals were noticed.
11. When the DNase digestion was skipped after the development, the signals were observed in all nuclei, although no primer was added to the reaction. As was discussed previously, this effect is very likely attributable to the repairing activity of r*Tth* polymerase used in this protocol, which may heal the nicks and gaps in the genomic DNA formed during the fixation and embedding processes ***(14)***.
12. Optimization of *in situ* RT-PCR needs to include many different control experiments to eliminate any false-positive or false-negative signals. Control experiments should be conducted in parallel on the same tissue section that is prepared for the experiments. For detecting false-positive signal reaction without labeled nucleotide, r*Tth* enzyme or primers could be performed. The *in situ* RT-PCR reaction with the antidigoxigenin–AP detection phase omitted can serve as an additional control for false-positives signals. As a control may use reaction mixture after RT-PCR step from under cover slip. Solution phase should be collected, next spotted on the nylon membrane, and hybridized with specific probe.
13. Cover the slide with a cover slip, avoiding formation of any bubbles. Make sure not to touch the section.
14. The parameters of the *in situ* RT-PCR reaction, especially the annealing temperature, should be optimized by applying the standard RT-PCR protocol in a solution phase (RNA isolated from the cucumber buds can be added). The reaction times applied were longer than usually used in a solution phase. It was a consequence of different conditions of *in situ* reaction. On the glass slide, the temperature fluctuation was much slower. A proper seal is very important to keep reaction concentrations consistent throughout the thermal cycling process. Concentrations of reagents are critical for a proper amplification.
15. The development time depends on the abundance of the mRNA and the structure of the cells. If a signal is not visible after 12 h, a fresh chromogenic substrate needs to be added. The slides can be stored in water at 4°C for up to 2 d. If slides are left longer than 2 d, the sections start to ruin and the stain diffuses.

16. Before covering the slides, remove all air bubbles, which can be detected as small, round bumps on their surface. If bubbles are present, try to cap the balsam oil.

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19

In Situ PCR for the Detection of Human Cytomegalovirus in Suspension Cells During the Latent Phase of Infection

Barry Slobedman

Summary

Cytomegalovirus latency depends on an interaction with hematopoietic cells in bone marrow and peripheral blood. The distribution of latent viral DNA and transcripts in these cells was investigated using methods based on polymerase chain reaction (PCR)-driven *in situ* hybridization (ISH) and reverse transcription (RT)-PCR-driven ISH. Using a conventional thermal cycler, latent viral DNA or transcripts were amplified within suspension cells. Amplified products were then detected by nonisotopic ISH on cells cytospun on glass microscope slides. During experimental latent infection of cultured granulocyte-macrophage progenitors, the viral genome was detected in more than 90% of cells. During natural infection, viral genomes were detected in 0.004 to 0.01% of mononuclear cells from granulocyte colony-stimulating factor mobilized peripheral blood or bone marrow from healthy seropositive donors. When evaluated by RT-PCR-ISH, only a small proportion of experimentally infected cells (approx 2%) had detectable latent transcripts. The application of PCR-ISH and RT-PCR-ISH has enabled the identification of the small percentage of bone marrow-derived mononuclear cells that become latently infected during natural infection and suggests that latency may proceed in some cells that fail to encode latent transcripts.

Key Words: Herpesvirus; human cytomegalovirus; latent infection; myeloid progenitor cell; suspension cells; *in situ* PCR; *in situ* RT-PCR; viral DNA; viral transcripts.

1. Introduction

Human cytomegalovirus (CMV) is a medically important β-herpesvirus carried by a majority of individuals, in whom it is a leading cause of opportunistic and congenital disease ***(1)***. Like other herpesviruses, primary infection by CMV leads to a life-long latency that is characterized by the maintenance of the viral genome without active infectious virus production. Periodically throughout life, virus reactivates from latency and is shed in bodily secretions, including saliva, urine, and breast milk. Although primary and reactivated infection

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remain largely asymptomatic in immunocompetent individuals, primary infection by CMV is a significant cause of serious congenital disease, leading to neurological damage in children. Reactivated infection is a major cause of disease in immunocompromised individuals, including patients with AIDS and allograft transplant recipients ***(1)***.

Peripheral blood (PB) and bone marrow (BM)-derived monocytes and granulocyte-macrophage progenitor cells (GM-P) are sites of CMV latency. The viral genome is maintained in cultured primary GM-Ps during experimental latent infection ***(2–5)*** and is detected in naturally infected PB- and BM-derived mononuclear cells from healthy, seropositive donors ***(6–9)***. Although the CMV genome is transcriptionally repressed during latent infection, a number of CMV latency-associated transcripts have been detected during latency ***(4,5,10)***. The ability of this virus to reactivate from a latent state contributes significantly to its success as a human pathogen, yet the tissue distribution of latent CMV has been poorly understood ***(11)***.

Quantitation of latent infection during natural infection has proved complicated primarily because of the need to apply polymerase chain reaction (PCR) methods to detect latent viral DNA and transcripts. Methods that rely on *in situ* hybridization (ISH) to enumerate RNA- or DNA-positive cells, particularly when combined with PCR amplification to increase sensitivity for low copy numbers, provide an accurate picture of the distribution of viral nucleic acids in host cells and tissues, although direct (i.e., non-PCR based) *in situ* detection of CMV DNA in latently infected cells has been reported ***(12)***.

This report describes the adaptation of PCR-driven ISH (PCR-ISH) and reverse transcription (RT)-PCR-driven ISH (RT-PCR-ISH) methods ***(13,14)*** to enumerate suspension cells harboring latent CMV genomes during experimental (**Fig. 1**) and natural latent infection (**Fig. 2**) or to enumerate latently infected cells expressing a class of CMV latency-associated transcripts from the major immediate early (IE1/IE2) region of the viral genome (**Fig. 3** ***[15]***). The procedure can be divided into two main steps: (1) *in situ* amplification of latent viral DNA or RNA, which is performed on cells in suspension using a conventional thermal cycler; and (2) the detection of amplified products within cells by nonisotopic ISH, which is performed on cells after being cytospun on glass microscope slides.

2. Materials

2.1. Preparation of Cells

1. Ficoll (Lymphoprep or similar).
2. HEPES-buffered saline solution (HBSS).
3. 1 *M* NH_4Cl stock.
4. 100 m*M* $KHCO_3$ stock.

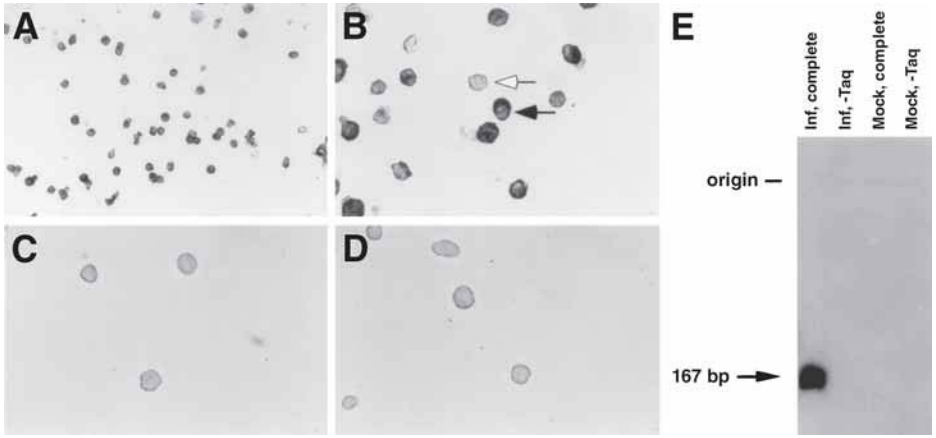

Fig. 1. Photomicrographs showing detection of CMV DNA in experimentally infected GM-P culture by PCR-ISH. Cells from CMV-infected **(A–C)** or mock-infected **(D)** cultures were subjected to PCR-ISH, except that *Taq* DNA polymerase was omitted from C. Filled arrow, CMV DNA-positive cell; and open arrow, CMV DNA-negative cell. Magnification is A, ×1280 and B-D; ×3200. **(E)** To confirm the specificity of the *in situ* PCR amplification of CMV DNA, total DNA was extracted from CMV-infected (Inf, complete) or mock-infected GM-Ps (Mock, complete) after PCR amplification with IEP3A and IEP3B or with the omission of *Taq* DNA polymerase (Inf, -*Taq* and Mock, -*Taq*) and subjected to DNA blot hybridization. Filter was probed with a ^{32}P-labeled probe derived from pON2810, washed, and exposed to X-ray film for 2 h. The predicted CMV-specific PCR product size of 167 base pairs is indicated.

2.2. In Situ *PCR Amplification*

All molecular biology reagents are from Invitrogen and Roche.

1. Phosphate-buffered saline (PBS).
2. Paraformaldehyde solution: 4% made in PBS.
3. 10X PCR buffer (Invitrogen).
4. 100 m*M* dATP stock.
5. 100 m*M* dCTP stock.
6. 100 m*M* dGTP stock.
7. 100 m*M* dTTP stock.
8. Gelatin: 1% solution.
9. RNase- and DNase-free water.
10. Mineral oil.
11. *Taq* DNA polymerase.
12. Glutaraldehyde-activated 3-aminopropyl-triethoxysilane (APES)-coated glass microscope slides (or similar coated slides compatible with ISH).

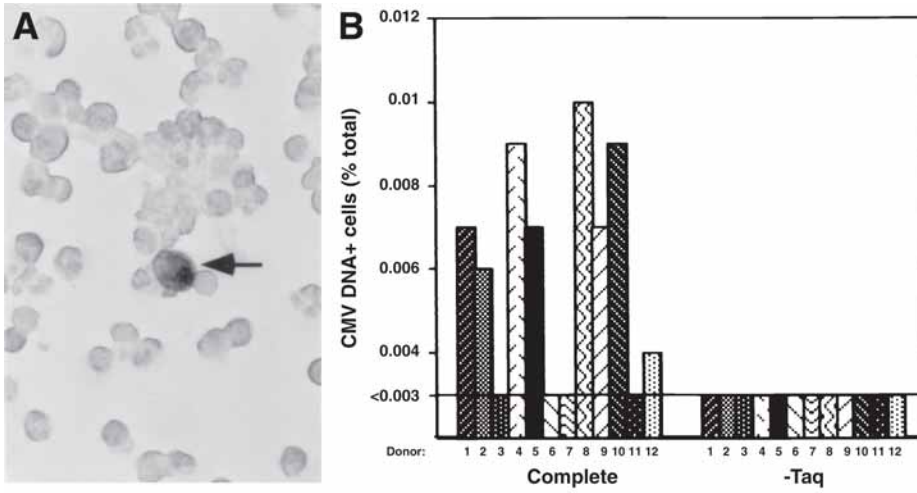

Fig. 2. **(A)** Photomicrograph showing detection of CMV DNA in naturally infected mononuclear cells by PCR-ISH. CMV DNA-positive cells are indicated by an arrow. Magnification is ×3200. **(B)** PCR-ISH determination of the percentage of CMV DNA-positive mononuclear cells from 12 separate granulocyte colony-stimulating factor-mobilized PB or BM donors. Cells were analyzed in the presence of the full complement of reagents for CMV DNA detection (complete) or with the omission of *Taq* DNA polymerase (-*Taq*).

2.3. In Situ *Detection of Amplified Products*

All molecular biology reagents are from Invitrogen and Roche.

1. Ethanol: 100%, 70%, and 50%.
2. 1% Glutaraldehyde solution.
3. Triton X-100.
4. Acetylation medium: 0.25% acetic anhydride in 0.1 *M* triethanolamine, pH 8.0.
5. Deionized formamide.
6. Yeast tRNA: 10 mg/mL stock.
7. Sonicated salmon sperm DNA: 10 mg/mL stock.
8. 5X Hybridization buffer.
9. Dithiothreitol (DTT): 100 m*M* stock.
10. RNase inhibitor: 10 U/mL stock.
11. Clean cover slips (50 mm × 22 mm).
13. Rubber cement.
14. SSC: 20X stock.
15. Washing solution: 0.1X SSC, 10 m*M* Tris-HCl, pH 7.5, 30% deionized formamide.
16. Buffer 1: 100 m*M* maleic acid, 150 m*M* NaCl. Adjust to pH 7.5 with NaOH.
17. Blocking reagent for nucleic acid hybridization (Roche).

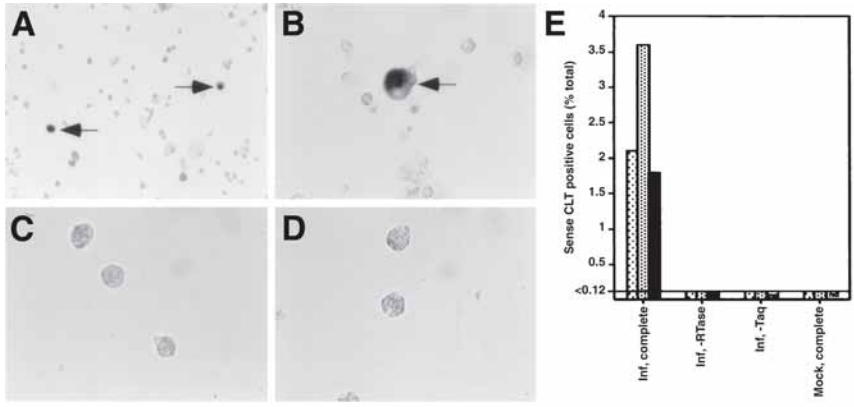

Fig. 3. Photomicrographs showing detection of CMV latency-associated transcripts (CLTs) from the IE1/IE2 region of the viral genome by RT-PCR-ISH in experimentally infected GM-P culture. Infected cells were subjected to RT-PCR-ISH (**A** and **B**), with controls omitting reverse transcriptase **(C)** or *Taq* DNA polymerase **(D)**. CLT-positive cells are arrowed. Magnification is A; ×1280 and B–D; ×3200. **(E)** RT-PCR-ISH determination of the percentage of CLT-positive cells from three separate GM-P cultures 2 to 3 wk after infection with CMV at a multiplicity of infection of 3. Virus (Inf) or mock (Mock) infected cells were analyzed in the presence of the full complement of reagents for sense CLT detection (complete) or with the omission of either reverse transcriptase (-RTase) or *Taq* DNA polymerase (-*Taq*).

18. Buffer 2 (prepare fresh): 1% (w/v) blocking reagent for nucleic acid hybridization in buffer 1.
19. Buffer 3: 100 m*M* Tris-HCl, pH 9.5, 100 m*M* NaCl, 50 m*M* $MgCl_2$.
20. Dimethylformamide: 100% and 70%.
21. Developing solution (30 mL): 135 µL of nitroblue tetrazolium chloride (NBT) stock (stock is 100 mg of NBT in 1.3 mL of 70% dimethylformamide), 105 µL of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) stock. Stock is 50 mg of BCIP in 1.0 mL 100% dimethylformamide (*see* **Note 1**). Made up to 30 mL with Buffer 3.

2.4. In Situ *Amplification and Detection of RNA Transcripts*

The following are needed in addition to those reagents listed previously.

1. DNase digestion mixture (200 µL):
   a. 1.2 µL of 1 *M* $MgCl_2$.
   b. 8 µL of 1 *M* Tris-HCl, pH 7.5.
   c. 8 µL of DNase (RNase free; 140 U/µL).
   d. 3 µL of RNase inhibitor (10 U/µL).
   e. 2 µL of 100 m*M* DTT.
   f. 177 µL of RNase- and DNase-free water.

2. RT buffer (190 µL):
   a. 5 µL of RNase inhibitor (10 U/µL).
   b. 20 µL of 100 m*M* DTT.
   c. 2 µL of 100 m*M* dATP stock.
   d. 2 µL of 100 m*M* dCTP stock.
   e. 2 µL of 100 m*M* dGTP stock.
   f. 2 µL of 100 m*M* dTTP stock.
   g. 5 µL of 5X RT buffer (as supplied with the reverse transcriptase).
   h. 5 µL of RT primer (IEP2D, 50 pmol/µL; *see* **Note 2**),
   i. 147 µL of RNase- and DNase-free water.
3. Reverse transcriptase (SuperScript II, Invitrogen).

3. Methods

3.1. Source of Cells

Detection of latent CMV infection was conducted using either (1) cultured primary human GM-Ps that had been latently infected in the laboratory with CMV (i.e., experimental latent infection) or (2) granulocyte colony-stimulating factor-mobilized PB or BM aspirates that had been collected from healthy donors (i.e., natural latent infection). BM or mobilized PB samples were layered over 15 mL of Ficoll (Lymphoprep or similar) and centrifuged for 15 min at 1000*g*. Cells were washed once in HBSS; treated with 155 m*M* NH_4Cl, 10 m*M* $KHCO_3$, pH 7.0, for 5 min to lyse any remaining red blood cells; and washed three times with HBSS before being processed further for PCR-ISH.

3.2. In Situ *Amplification and Detection of Latent CMV DNA in Myeloid Progenitor Cell Suspensions by PCR-ISH*

3.2.1. In Situ *PCR Amplification*

1. Wash cells (*see* **Note 3**) three times in PBS (*see* **Note 4**).
2. Resuspend cells in 4% paraformaldehyde (*see* **Note 5**) and fix for 30 min at room temperature.
3. Wash cells 3X in PBS (*see* **Note 6**). For the final wash, transfer cells to 0.5 mL of PCR tubes, and split cells as appropriate to include a –*Taq* DNA polymerase control.
4. Resuspend cells in 48 µL of PCR mixture to make 192 µL (enough for four reactions):
   a. 20 µL of 10X PCR buffer (with 15 m*M* $MgCl_2$).
   b. 4 µL of 10 m*M* dATP.
   c. 4 µL of 10 m*M* dCTP.
   d. 4 µL of 10 m*M* dGTP.
   e. 4 µL of 10 m*M* dTTP.
   f. 2 µL of 1% gelatin.

g. 4 µL of forward primer (IEP3A, 50 pmol/mL; *see* **Note 7**).
h. 4 µL of reverse primer (IEP3B, 50 pmol/mL; *see* **Note 7**).
i. 146 µL of RNase- and DNase-free water.

5. Overlay with mineral oil and place into a conventional PCR thermocycler.
6. Denature for 8 min at 94°C, with 2 µL of *Taq* DNA polymerase added after the first 4 min (*see* **Note 8**). Then continue the denaturation for 4 min before thermocyling as follows for 30 cycles:
   a. 94°C, 2 min.
   b. 58°C, 2 min (as appropriate for gene specific primers).
   c. 72°C, 5 min.
7. After the cycling, cool to 4°C.
8. Wash cells 1X in PBS. Resuspend in PBS.
9. Cytospin onto glass microscope slides coated for ISH applications, for example, glutaraldehyde-activated APES-coated slides ***(16)***.
10. Air-dry for 30 min.

3.2.2. In Situ *Detection of Amplified Products*

1. Fix the cells in 4% paraformaldehyde (*see* **Note 5**) for 30 min and then wash twice for 5 min in PBS.

The next two steps are only required if storing the slides at this point is desired. If continuing straight on with the procedure, omit the following two steps:

2. Wash twice in 50% ethanol (ETOH), once in 70% ETOH, once in 100% ETOH (5 min each wash), and air-dry (*see* **Note 9**). Slides can be stored at this point.
3. Rehydrate through graded ETOHs (100%, 70%, 50%) to PBS.
4. Fix 0.1% glutaraldehyde (in PBS) for 30 min at 4°C (*see* **Note 10**).
5. Wash twice for 5 min in PBS.
6. Permeabilize cells in 1% Triton X-100 (in PBS) for 2 min.
7. Wash four times briefly in PBS.
8. Refix 0.1 % glutaraldehyde for 15 min at 4°C.
9. Acetylate in acetylation medium for 10 min (*see* **Note 11**).
10. Wash twice for 5 min in PBS.
11. Dehydrate through graded ETOHs (50%, 70%, 100%). Air-dry (*see* **Note 12**).
12. Prepare hybridization mixture in an RNase-free 1.5-mL tube. For 100 µL, prepare:
    a. 50 µL of deionized formamide.
    b. 5 µL of tRNA (10 mg/mL).
    c. 5 µL of sonicated salmon sperm DNA (10 mg/mL).
    d. 4 µL of digoxigenin (DIG)-labeled riboprobe (*see* **Note 13**).

    Heat to 65°C for 5 min, then cool on ice for 2 min. Next, add:
    a. 20 µL of 5X hybridization buffer.
    b. 1.5 µL of 100 m*M* DTT.
    c. 2 µL of RNase inhibitor (10 U/mL).
    d. 12.5 mL of RNase- and DNase-free water.

13. Carefully pipet 18 µL of hybridization mixture onto each dried cell spot.
14. Cover with clean cover slip (*see* **Note 14**) and seal edges completely with rubber cement to prevent drying out of the cell spot.
15. Denature target DNA by placing slides onto a heating block at 98°C for 8 min before cooling on ice for 2 min (*see* **Note 15**).
16. Reseal cover slips and hybridize at 47°C overnight.
17. Carefully remove the rubber cement and cover slip (*see* **Note 16**).
18. Wash as follows (with gentle shaking):
    a. 30 min in 2X SSC at room temperature (1000 mL).
    b. 30 min in 0.1X SSC at room temperature (1000 mL).
    c. 30 min in washing solution, 47°C (50 mL in Coplin jar).
    d. 15 min in 0.1X SSC at room temperature (1000 mL).
19. Wash 5 min in buffer 1 (50 mL in Coplin jar with gentle shaking).
20. Block for 30 min in buffer 2 (50 mL in Coplin jar with gentle shaking).
21. Wash twice for 15 min in buffer 1 (50 mL in Coplin jar with gentle shaking).
22. Wash once for 5 min in buffer 3 (50 mL in Coplin jar with gentle shaking).
23. Place slides into a fresh Coplin jar containing 30 mL developing solution (*see* **Note 17**).
24. Incubate in the dark (without shaking) until signal develops (15 min to 6 h; *see* **Note 18**).
25. Stop developing reaction by washing five times briefly with double distilled water (*see* **Note 19**).

3.3. In Situ *Amplification and Detection of Latent CMV RNA in Myeloid Progenitor Cell Suspensions by RT-PCR-ISH*

1. Wash cells (*see* **Note 3**) three times in PBS (*see* **Note 4**).
2. Resuspend cells in 4% paraformaldehyde (*see* **Note 5**) and fix for 30 min at room temperature.
3. Wash cells three times in PBS (*see* **Note 6**).
4. Resuspend cells in 200 µL of DNase digestion mixture.
5. Incubate overnight at 37°C.
6. Wash cells three times in PBS.
7. Resuspend cells in 95 µL of reverse transcription buffer.
8. To each 95 µL of mix + cells, add either 5 µL of reverse transcriptase (SuperScript II) or, as a negative control, add 5 µL of RNase- and DNase-free water.
9. Incubate at 42°C for 4 h.
10. Wash cells three times in PBS. For the final wash, transfer cells to 0.5-mL PCR tubes, and split cells as appropriate to include a -*Taq* polymerase control.

Continue with PCR amplification and detection as described from **step 4** onward in **Subheading 3.2.**, with the following modifications: (1) Instead of primers IEP3A and IEP3B, use primers IEP2D and IEP1G for the PCR amplification, and (2) the detection of *in situ*-amplified products uses a different DIG-labeled riboprobe (*see* **Notes 20** and **21**).

4. Notes

1. Store stock solutions of NBT and BCIP at –20°C.
2. Primer IEP2D 5'-CAGGATTATCAGGGTCCATCTTTCTCTTGG-3' and IEP1G 5'-ATAGCAGAGCTCGTTTAGTGAACCG-3' are derived from within exon 2 or exon 1, respectively, of the CMV IE1/IE2 region ***(17)***.
3. There will be some loss in cell number during, for example, spins and washes. Although this procedure can be conducted with significantly less cells, a starting of greater than 10^5 cells per PCR is recommended.
4. Ensure that washing buffer is magnesium-free PBS because the presence of magnesium may significantly alter the subsequent PCR amplification step.
5. Only use freshly prepared 4% paraformaldehyde solution (room temperature) made in PBS. Dissolving paraformaldehyde requires raising the pH with 10 *N* sodium hydroxide. After the paraformaldehyde has completely dissolved, pH can be returned to neutral with concentrated HCl.
6. All washes of cells are performed in 0.5-mL tubes (or similar), with cells being pelleted using a swing-out rotor. If a 0.5-mL tube rotor is not available, the 0.5-mL tubes can be placed into a larger tube, for instance, 15- or 50-mL conical centrifuge tubes, and centrifuged using a larger rotor. If PCR is to be performed using a 96-well format PCR block, then the same steps can be conducted in smaller PCR tubes. It is worth doing trial centrifugations to ensure that thin walled PCR tubes will not fracture during the washing steps.
7. Primers IEP3A 5'-GTGACCAAGGCCACGACGTT-3' and IEP3B 5'-TCTGCCA GGACATCTTTCTC-3' are derived from within exon 3 of the IE1/IE2 region of CMV *(3)*. Primer design for PCR amplification: The PCR product size for the detection of CMV DNA is 167 bp. Using this method, significantly larger PCR products (e.g., 1 kb) were found to be very difficult to successfully amplify *in situ*.
8. After 4 min of denaturation at 94°C, pause the machine and add 2 µL of *Taq* DNA polymerase. Continue the denaturation for 4 min before thermacyling.
9. After air-drying, slides can be stored in a slide box in at –20°C. The slides can be stored this way until being ready to do the ISH.
10. A staining dish positioned in ice works well for this step.
11. Prepare immediately before use in water with vigorous mixing.
12. Air-dry in a dust-free environment. The cell spots are now ready for the addition of the hybridization mixture. The hybridization mixture can be prepared while the cell spots are drying.
13. Detection of *in situ*-amplified PCR products was performed using a nonisotopic DIG-labeled riboprobe generated from a genomic clone, pON2810, which consists of a 2.2-kb *Alw*NI restriction fragment from the IE1/IE2 region of human CMV strain AD169 cloned into the SmaI site of pBluescript KS (+/–).
14. Cover slips often are contaminated with oils from the manufacturing process and are therefore best thoroughly cleaned before use with an acid wash or detergent solution.
15. After heating to 98°C for 8 min, immediately transfer the slides to a precooled surface for 2 min. A thin metal plate placed onto a bed of ice works well for this cooling step.

16. When removing the cover slip, it is important to not disrupt the cells on the slide or allow any of the hybridization solution to dry onto the slide. Thus, after removing the rubber cement from around the cover slip, place the slide with cover slip still in place into a slide rack and immerse in the first wash buffer. The cover slip can then be gently removed with a pair of fine forceps.
17. Developing solution should be made fresh, immediately before use.
18. Signal development can be checked periodically by removing from developing solution and very briefly examining the slides under a light microscope (with the lamp intensity lowered, as the developing solution is somewhat light-sensitive).
19. After stopping the reaction, slides should be left in double distilled water or cover slipped with a water-based mounting medium. Do not use alcohol or other solvent-based mounting media because they will remove positive staining.
20. *In situ*-amplified RT-PCR products were detected by ISH using a DIG-labeled riboprobe derived from pON2501, which contains a 1.1- kb *Eco*RV/*Spe*I complementary DNA fragment from the IE1/IE2 region of CMV strain AD169 cloned into the ClaI/SpeI site of pBluescript KS (+/–).
21. Primer design for reverse transcription: The design of the RT primers was such that the RT step was across a large spliced region (intron). This was done to bias the PCR reaction in favor of amplification of the complementary DNA rather than a much larger genomic template, in addition to the DNase digestion.

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20

Development and Adaptation of the PRINS Technology

An Overview

Franck Pellestor

Summary

The advent of molecular genetic techniques has brought forth new procedures for *in situ* chromosomal analysis. One of these techniques is the primed *in situ* labeling (PRINS) procedure, which constitutes a fast and efficient alternative to conventional fluorescence *in situ* hybridization for nucleic acid detection. Based on the use of chromosome-specific primers, the PRINS method combines the high sensitivity of the PCR reaction with the cytological localization of DNA sequences. Since its introduction, the PRINS protocol has been optimized, and numerous applications have been developed. The technique has thus proved to be a useful tool for *in situ* chromosomal screening, and has become a simple and efficient complement to conventional and molecular cytogenetic methods.

Key Words: PRINS; primer; α-satellite sequences; chromosomal aneuploidy; unique sequence detection.

1. Introduction

The advent of molecular genetic techniques has led to the development of a new approach for cytogenetic screening in interphase nuclei, called interphasic cytogenetics. This strategy has become an important tool for chromosome research and cytogenetic diagnosis because it bypasses the culture phase required to generate metaphases in conventional cytogenetic procedures and to perform analysis on rarely dividing or nondividing cells that previously were not available to cytogenetic investigations. One of these techniques is the primed *in situ* labeling (PRINS) procedure, which constitutes an efficient alternative to conventional fluorescence *in situ* hybridization (FISH) technique for chromosomal screening. Both FISH and PRINS were developed around the same time ***(1,2)***. Because it is relatively easy to implement and perform and because a large variety of DNA probes and *in situ* hybridization kits are

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commercially availability, FISH has become the technique of choice for chromosomal investigations in both medical institutions and research centers. During the past decade, the parallel development of *in situ* detection systems and sophisticated microscopy has resulted in the introduction of multiple advanced FISH techniques, which have revolutionized the practice of human cytogenetic ***(3,4)***.

Compared with such a well-established technology and its innovative techniques, the PRINS procedure might appear of minor interest and of limited usefulness. However, the PRINS reaction presents several advantages, such as rapidity, high sensitivity, and high specificity, making the PRINS technique an attractive complement to FISH for *in situ* aneuploidy detection and study of subtle chromosomal rearrangements.

2. Principles and Methods of PRINS

The PRINS reaction combines the high sensitivity of the polymerase chain reaction (PCR) with the cytological localization of specific DNA sequences. The key to the PRINS technique is the use of unlabeled, short, and specific oligonucleotides. Oligonucleotides are annealed *in situ* to complementary DNA targets on denaturated chromosome spreads, nuclei, or tissue sections and then act as primers for chain elongation catalyzed by a *Taq* DNA polymerase in the presence of free nucleotides. The visualization of generated fragments results from the incorporation of one labeled nucleotide.

2.1. Definition of PRINS Primers

In their original report, Koch et al. ***(2)*** reported the *in situ* labeling of consensus centromeric repeated sequences. Subsequent studies identified oligonucleotides useful in identifying each type of repeat DNA sequences spreading over the human genome, that is, satellite, telomeric, or Alu DNA sequences ***(5)***. A great advantage of primers is their ability to differentiate between closely related sequences. This feature has been used for generating chromosome-specific primers from the α-satellite DNA motif ***(6,7)***. α-Satellite (or alphoid) DNA is a family of tandemly repeated sequences present at the centromere of all human chromosomes. These centromeric repeats are made up of a variable number of monomeres of 171 base pairs (bp) in length and are organized as α-satellite subfamilies. The DNA sequences of momomeres slightly deviate among subfamilies and individual chromosomes. The chromosome specificity of PRINS is based on the use of primers generated from these chromosome-specific α-satellite DNA sequences. The complementation process between the primer and its centromeric target will be so specific that a simple mismatch between the 3'-end of the primer and the genomic sequence will prevent initiation of the *in situ* elongation by the *Taq* DNA polymerase. Thus, it has been

possible to define specific α-satellite primers for most of the human chromosomes, involving some chromosomes undistinguishable by conventional FISH with centromeric probes. This is the case with chromosomes 13 and 21, which share 99.7% homology in their α-satellite DNA sequences ***(8)***. To date, specific primers have been defined for 20 human chromosomes (*see* Chapter 6). Efficient primers have also been defined for *in situ* labeling of telomeres and studying variant telomeric repeats ***(9,10)***.

Using an automatic DNA synthesizer, both the preparation and the purification of oligonucleotides are now fast and rather inexpensive. The length of the PRINS primers ranges from 18 to 35 nucleotides. Compared with the size of DNA repetitive probes (250–600 bp), this small size greatly facilitates their *in situ* accessibility to their genomic target sequences, which is particularly significant in cells with highly condensed nuclei, such as spermatozoa. The use of oligonucleotide primers eliminates the need for probe preparation and can also circumvent the lack of resolution of DNA probes (approx 5–10 kb in interphase nuclei), because every known DNA sequence can be a potential primer source for PRINS reaction. As an alternative to oligonucleotides, cloned probes fragmented by restriction enzyme digestion also can been used as primers ***(11)***.

2.2. Coming of Age and Advanced PRINS Procedures

Initially, PRINS reactions were performed either on a hotplate or a water bath. The required changes of temperature were achieved by moving the sides as often as needed. The weakness of these methods was in the lack of stringency of primer annealing and high background. These procedures did not allow precise and durable temperature control. Indeed, although the range of *Taq* DNA polymerase activity is large enough, the optimization of the annealing and its stringency to increase specificity need accurate temperature control. The protocol has been considerably improved and simplified by the introduction of programmable temperature cyclers equipped with a flat plate block. With this equipment, the precision of temperature control may reach 0.2°C and the required temperature changes are both easy to program and rapidly conducted. The use of automatic thermocyclers allows an optimization of both annealing and extension conditions. Thus, semiautomatic PRINS protocols were developed offering a high reproductibility in labeling reaction. An additional improvement was the direct use of various fluorochromes and the introduction of the multiple-color PRINS reaction ***(7,12)***, allowing the sequential labeling of several chromosomal targets on a same cell preparation. By incorporating directly fluorochrome-labeled nucleotides into newly synthetized DNA fragments, the turnaround time of the PRINS reaction was reduced to no more than a few minutes, leading to the development of ultrafast PRINS protocols ***(13)***. Attempts to increase sensitivity also involved the use of serial cycles

of PRINS reactions for accumulating labeled copies of the target sequence at the site of synthesis ***(14,15)***. However, this process, called cycling PRINS, also could alter the specificity of the signal and quality of the chromosome preparation ***(16)***.

In practice, a PRINS mix is prepared in a final volume of 50 µL containing the oligonucleotide (50–250 p*M*); the nucleotide mixture, including a labeled dUTP (biotine, digoxigenine, fluoresceine, coumarine, rhodamine, cyanine, etc.), the *Taq* polymerase buffer; and 1 to 2 U of the enzyme. Because they are unlabeled, high amounts of primers can be used in PRINS reaction without inducing background signals. Both chemical and thermal denaturations of the preparation slide can be used. Thus, a classical PRINS procedure consists of two programmed steps (annealing at the specific annealing temperature of the used primer, and nucleotide chain elongation at 72°C) in case of preliminary chemical denaturation (in 70% formamide, 2X standard saline citrate [SSC] at 72°C), whereas the thermic denaturation of the preparation is directly involved in the PRINS reaction, then consisting of three programmed steps (denaturation at 94°C, annealing, and elongation). Usually, slides and cover slips are not sealed. Both the volume of the mix and the short incubation time prevent the slide from drying during the reaction. In conventional multitarget PRINS reaction, a blocking step is used between each chromosome-specific PRINS reaction to prevent the mixing of labeling and to ensure a good recognition of each targeted site. The slide is thus treated at 37°C with a dideoxynucleotides mix and Klenow enzyme, which block the free 3'-ends of the elongation fragments generated by the previous PRINS reaction. Detection of the labeling sites is performed by fluorescence microscopy. Initial PRINS procedures used primers labeled with biotine or digoxigenine, and the detection of the labeling sites required immunocytochemistry procedure for detecting reporter molecules. Now, most of the PRINS reactions are performed using directly fluorochrome-labeled nucleotides, which allows the direct visualization of targeted sequences on fluorescence microscope.

Recently, a new multicolor PRINS protocol has been reported, allowing the performance of ultra-rapid detection of several chromosomes only by mixing the different fluorochromes during the chain elongation reaction ***(17)***. In this sequential procedure, each PRINS reaction consists of a unique short step (4–6 min) for annealing and elongation of each chromosome-specific primer. This ingenious strategy, which eliminates the intermediate blocking reaction, greatly simplifies the PRINS protocol and facilitates its adaptation to various cytogenetic applications (*see* Chapter 1 and 6).

Compared with the conventional FISH technique, the PRINS method has proven to be equally accurate for detecting chromosome, quicker, and less expensive. Werner et al. ***(18)*** estimated that the PRINS reaction costs approximately one-tenth as much as FISH.

3. Applications of PRINS

The PRINS procedure combines several features that make it very attractive for a number of cytogenetic purposes. Although PRINS initially was limited to the detection of repeat sequences, numerous applications of PRINS technology have been developed in mammals, fish, insects, and plants, demonstrating that PRINS could be easily adapted to various types of cells.

In *Drosophilia*, PRINS was used for gene mapping on polytene chromosomes ***(19)***, and in fish, the localization of Alu repeat and nuclear organizer regions was performed by PRINS ***(20,21)***. In plants, PRINS was used for labeling telomeres ***(22)*** and gene clusters ***(23)***. The technique also was adapted to suspensions of bean chromosomes to facilitate the flow sorting and karyotyping of similar-sized chromosomes ***(24)***. In mammalian cells, a large variety of PRINS applications have been described, and the technique has been integrated into various protocols of research. For instance, Russo et al. ***(25)*** used PRINS for studying mechanisms of aneuploidy in mouse splenocytes. Because of its high sensitivity, the PRINS technique was also chosen for telomeric studies in various species ***(26,27)***.

In humans, PRINS method has been successfully adapted not only in the assessment of aneuploidy in lymphocytes, amniocytes, and preimplantation embryos ***(28–30)*** but also in the analysis of structural aberrations, such as translocations, marker chromosomes, and ring chromosomes ***(31)***. More recently, the PRINS protocol has been adapted to detect fetal cells among separated maternal nucleated cells from peripheral venous blood of pregnant women, demonstrating the efficiency of the technique for noninvasive prenatal chromosome analysis ***(32,33)***. As the PRINS reaction with Alu primers gives high quality R-like banding on human chromosomes, the procedure has been adapted for the cytogenetic screening of somatic hybrid cell lines ***(34)*** and identification of euchromatin in aberrant short arms of acrocentric chromosomes and small ring chromosomes ***(35)***. Further applications have been found in tumoral cytogenetics. To date, preliminary studies involve the detection of host cells after sex-mismatched bone marrow transplantation in patients grafted for leukemia and the cytogenetic screening of tumoral cell lines ***(36)***. The PRINS technique also can be performed in both frozen and paraffin-embedded tissue sections or to localize RNA within cells, which generates new possibilities for cytogeneticists and pathologists ***(37)***. All these applications point out the potential efficiency of PRINS method for diagnostic use.

Taking advantage of the high sensitivity and specificity of PRINS reaction, some laboratories have successfully adapted the technique to the *in situ* detection of RNA ***(38)*** and the quantitation of RNA species in cell suspensions by flow cytometry ***(39)***. In the same way, Andersen et al. ***(40)*** use a variation of PRINS (the self-PRINS) to study the methylation status of CpG islands in

human chromosomes. By using variant telomeric primers, Krejci and Koch ***(41,42)*** clearly demonstrated the superiority of PRINS for measuring telomeric length and identifying polymorphic telomeric repeats. Studies have also shown that PRINS and FISH could be used in concert for simultaneous detection of chromosome targets ***(43,44)***.

One of the most remarkable and efficient applications of the PRINS technique has been conducted on human spermatozoa. The adaptation of PRINS technology to male gametes constituted an interesting challenge because of the particularities of sperm nucleus in term of genomic compaction and accessibility of DNA sequences. The PRINS methodology has been combined with NaOH treatments, which allows the simultaneous decondensation and denaturation of sperm nuclei. In PRINS reaction, the decondensation of the sperm head is a less limiting factor than in FISH because of the small size of the oligonucleotide primers. This facilitates their penetration into sperm nuclei and their access to the genomic target sequences, resulting in a more efficient and rapid labeling of sperm nuclei. Using standart PRINS technique, diploidy and disomy frequencies have been estimated for most of the human chromosomes in sperm samples from several men ***(45)***, and studies of meiotic segregation pattern were performed in sperm samples from reciprocal translocation carriers ***(46)***. Recently, the new multicolor PRINS protocol has been adapted successfully on human spermatozoa ***(47)***, as well as on human oocytes and blastomeres ***(48)***.

The adaptation of PRINS technique to the *in situ* detection of unique sequences has constituted an important challenge because this procedure could allow to rapidly map genes on chromosomes by simply using synthetic oligonucleotides derived from sequenced DNA. The advantage of the PRINS approach is that it does not rely on the possession of a cloned probe for the target. As long as the sequence of the gene is known, oligonucleotides can be synthesized for use as primers. Several teams have worked on this new application of PRINS. Preliminary results have been obtained on porcine chromosomes where unique sequences as short as 100 to 300 bp were localized by using pairs of specific primers ***(49)***. More recently, the procedure has been optimized to detect single copy loci on human chromosomes. Thus, Kadandale et al. ***(50)*** first localized the *SRY* gene *in situ*, and several further reports showed that PRINS could be used for gene mapping ***(16,51)*** but also for the diagnosis of microdeletion syndromes and cancer abnormalities (***52,53)***, opening news and promising perspectives for PRINS in the field of physical mapping.

4. Conclusion

PRINS has emerged as a research technique, and since its introduction, the technique has undergone many stages of development to improve the sensitivity, the efficiency, and the versability of the process. The PRINS technique has moved from research benches to diagnostic laboratories, and numerous studies

have demonstrated that PRINS is as reliable and more efficient than FISH for detecting chromosomes in metaphase and interphase nuclei. With the development of rapid and simplified protocols producing reliable and reproducible results, PRINS has become a powerful tool for cytogenetic investigations and diagnosis. During the last few years, PRINS has progressed from the labeling of repeat sequences to the use for the detection of unique sequences. New innovative variations to PRINS, such as padlock probes or rolling circle PRINS (*see* Chapter 5), have been introduced ***(54,55)***, which leads to think that the PRINS technology could provide an efficient complement to FISH and PCR in various diagnostic and research situations.

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21

Latest Developments in *In Situ* PCR

Omar Bagasra and Twaina Harris

Summary

Since the first publication on the method of *in situ* polymerase chain reaction (PCR), several thousand research papers have appeared in peer-reviewed journals describing various findings based solely on the application of this method or combined with other more robust methods, including solution-based PCR, immunohistochemistry, Southern blot, etc. A few years after the advent of PCR, several investigators developed *in situ* PCR methods that differed considerably from each other with regard to tissue preparations, fixation, mounting of slides, reverse transcription technique, primer design, target selection, size, and amplicon size, and thermocycler designs and the use, among many other fine details. This chapter describes the detail procedures that are used in the author's laboratory. It also discusses the variations and modification that can be used for the specific needs of an investigator. This protocol should serve as a primer for the investigators, and each researcher must use his or her variation according to their needs.

Key Words: *In situ* PCR; PCR; primer design; reverse transcription; target selection; tissue preparation; thermocycler.

1. Introduction

The solution-based polymerase chain reaction (PCR) method for the amplification of defined gene sequences has proven to be a valuable tool not only for basic researchers but also for clinical scientists. By using even a minute amount of DNA or RNA and choosing a thermostable enzyme from a large variety of sources, one can enlarge the amount of the gene of interest, which can be analyzed, cloned, or sequenced in a very short amount of time. Thus, genes or segments of gene sequences present only in a small sample of cells or a small fraction of mixed cellular populations that can be examined ***(1)***.

The ability to identify individual cells expressing or carrying specific genes of interest in a latent form in a tissue section under the microscope provides a

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great advantage in determining various aspects of normal, as opposed to pathological conditions. By using the proper primers for genes that are expressed by certain histological cell types, one can potentially determine the origin of metastatic tumors by performing reverse transcriptase (RT)-based *in situ* PCR ***(2)***.

Our laboratories have been using *in situ* PCR techniques for several years and we have developed precise, sensitive protocols for both RNA and DNA that have been proven to be reproducible in multiple double-blinded studies ***(3–12)***. One can use this method for the amplification of both DNA and RNA gene sequences. By the use of multiple labeled probes, one can detect various signals in a single cell ***(12,13)***. In addition, under special circumstances, one can perform immunohistochemistry, RNA, and DNA amplification at a single-cell level (the so-called "triple-labeling" ***[9,14,15]***). This chapter presents a detailed protocol currently used in our laboratory.

Before one attempts to conduct *in situ* PCRs on tissues or cells, we strongly recommend that all investigators first optimize the PCRs in solution to get the primers, probes, and the incubation temperatures all working right before throwing the additional complication of cellular matrices into the mix. Investigators universally report that amplification reactions are more problematic in tissues and cells, and virtually no one gets *in situ* amplification correctly the first time they try if they are working alone. Unfortunately, troubleshooting these early reactions is usually very difficult because there are so many variables involved.

Solution-based reactions are relatively easy, and one can have success rather quickly—often on the first try. Then the reactions can be quickly optimized so that the results will be even better, and optimized parameters almost always transfer more successfully to the *in situ* protocols. Solution-based reactions also can serve as controls for *in situ* PCR.

However, before DNA or RNA can be amplified in a solution, it must be extracted from the cellular matrix in some manner. There are several well-established extraction procedures. Readers are advised to follow the precise extraction methods from any of the well-established molecular biology methods (i.e., Short Protocol in Molecular Biology or Molecular Cloning ***[16–19]***). In addition, several manufacturers of molecular biology products sell extraction kits that can be used for the specific purposes for which they are intended.

2. Review of the PCR Technique

PCR is now one of the most widely used procedures in almost every aspect of biological sciences. Readers who are still new to PCR should consult any of the numerous articles, animation and simulation available on almost all the search engines (i.e., Google, Yahoo, etc.). Our favorite is from the Cold Spring Harbor Laboratory (http://www.dnalc.org/).

2.1. Reverse Transcription: Making Complementary DNA From RNA

Of course, DNA serves as the primary "library" of genetic information for any organism, and it resides primarily in the nucleus of a cell (at least with eukaryotes). RNA serves as the working transcript of genetic information, and it is from RNA templates that proteins are eventually synthesized in the ribosomes. RNA molecules tend to be much smaller than DNA molecules and they move freely about the cell, and having shorter lifetimes (RNA is sometimes described as being like a photo copy of an individual page of an organism's book of life, which can be discarded after they have done their job). RNA molecules almost always are single stranded and somewhat less stable physically and chemically than double-stranded DNA. Also, they are quickly degraded by ubiquitous enzymes called "RNases"—these continuously get rid of used RNA and recycle it in vivo but can also quickly chew up an investigator's target RNA, especially after a cell has died and before it is fixed.

In all life forms, whether prokaryotic or eukaryotic, the direction of transcription is almost always from DNA to RNA to protein—the permanent record to the working copy. Very rarely does transcription occur in the reverse direction. However, some RNA viruses—namely retroviruses—contain an enzyme called reverse transcriptase that can do just that, make DNA copies (called complementary [c]DNA) from RNA originals. The enzymes from various types of retroviruses have been isolated and cloned and are available in many different variations, and their activity can be exploited to make cDNA from RNA in vitro. This ability can be put to great advantage with *in situ* PCR because it allows an investigator to indirectly amplify RNA within cells so that it can be readily detected even when the target is in very low abundance. This amplification is achieved by first converting single-stranded RNA into double-stranded cDNA with the RT enzyme, then PCR is conducted on the cDNA copy to amplify the sought-after signal (so far, PCR works only with DNA, not with RNA). The result is that an investigator can determine whether a specific gene is actively expressed within a cell by determining whether RNA copies of the gene are present within the cellular structure (there is an especially elegant manner to amplify RNA in a single step, discussed separately in **Subheading 4.5.**).

All RT enzymes exhibit at least four specific enzymatic activities, namely reverse transcription, a RNA-dependent DNA polymerase activity, ribonuclease H (RNase H) activity and integration capability (we do not use the last one for our purpose). The reverse transcription maneuver allows the enzyme to use any RNA as a template to be copied, provided that the action is initiated by an annealed primer at the beginning of the target sequence. The copy being made is always DNA (cDNA). The ribonuclease H activity serves to peel away the RNA template from the newly created RNA–DNA hybrid, and then the enzyme cuts down the original RNA molecule into tiny bits. The single-stranded cDNA

molecule that remains is manipulated by the enzyme once again; this time, another domain of RT enzymes acts like DNA polymerase and weaves a complementary strand along single-stranded cDNA to make a double stranded cDNA molecule.

The different versions of the RT enzyme each have slightly different characteristics as the result of the different origins of the enzymes. Each was derived from a specific retrovirus that has evolved a particular capsid protein to provide a structural milieu within which the enzyme can do its work. However, in the artificial environment of an in vitro experiment, there are no capsid proteins available, and hence no optimized milieu for the enzyme. Therefore, the investigator must take special care in using RT enzymes; otherwise, various untoward biochemical characteristics of the enzyme can be summoned forth.

In particular, RT enzymes in vitro have a tendency to give up on RT too early and start chopping up the RNA template before transcription is complete. This destroys the RNA signal before a complete cDNA copy is synthesized. An investigator minimizes this effect by optimizing reagents, particularly manganese ion and a specific detergent that substitutes for some of the characteristics of the capsid proteins. Generally, vendors of RT enzymes supply an optimized buffer solution containing proper concentrations of the manganese ion and detergent (be certain to always use the optimized buffer with each RT enzyme).

Last but not least, there are several newer RT enzymes available with special characteristics. One, called *rTth*, is available from Perkin Elmer and can serve not only as the RT enzyme but also as the thermostable DNA polymerase for PCR, allowing RT and PCR reactions to be conducted in the same buffer in one thermal cycling regime. Few other RTs with similar characteristics have been isolated from prokaryotes, which survive at very high temperatures. Many RTs have other qualities, including proofreading ability, longer life-span, and the ability to reverse transcribe longer messenger (m)RNAs. Investigators ought to search for the right enzymes, which is best suited for their tasks. One way to look for various reagents is to log onto http://www.sciquest.com to search for various reagents.

Currently, we are using a ready to go kit from Amersham Biosciences. There are several kits available in the market and a researcher can use their own judgments in utilizing one of these kits that is suitable for their particular project.

2.2. How to Design Primers

One of the most important keys to performing PCR successfully is designing a proper primer pair and then optimizing the annealing temperature so that amplification can proceed smoothly. Fortunately, numerous resources and computerized tools are available to assist in these matters, and an investigator is well advised to pay particular attention to these details early in the development of an experimental protocol, for it will save much agony later on.

2.2.1. Primers for DNA Targets

Primers are synthetic oligonucleotides, typically between 18 and 22 bases in length. Some might consider an 18-mer primer a little short with too much chance that random annealing occurring. Others would argue that a 22-mer primer is extravagantly long. It is our experience that primers in this range work effectively.

Among the four primary nucleotides (i.e., G, A, T, and C) in DNA and primers, two are "stickier"—G and C—than the others are, as they form six hydrogen bonds between them (A and T only form four). This varying character can affect the performance of the primer substantially. Therefore, it is desirable to have G and C nucleotides at the 3' (downstream) end of the primer because this will facilitate annealing. However, one does not want a triple GGG or CCC at the 3'-end because this combination is too sticky, nor should one have AAA or TTT. Rather, the ideal sequence is to have two GC nucleotides followed by an AT type at the 3'-end, like GCT or GGA. The overall GC-content of the primer should be between 45 and 50%.

The two primers should be designed so that they have approximately the same annealing temperature. They also should be designed so that they do not form intrastrand or interstrand base pairs, which may result in hairpin formation. Single strands of DNA can twist and form loops in such a way that part of the primer can anneal to a target, with another part annealing to a spot on the target tens or hundreds of bases away from the first. One must be careful to select sequences that have negligible complementary regions, even in short fragments, in the larger segment of the DNA intended as the target. Primers that anneal to multiple regions of the target will neither identify the targeted sequence, nor will they extend the target properly.

Furthermore, primers should never be complementary to one another, particularly around their 3'-ends. If they complement in this region, they often anneal to each other and thus form "primer dimmers." These get extended by the polymerase into double-stranded DNA the length of two primers combined, minus the length of the complementary region. This is always undesirable, because it consumes the primers and greatly lowers the efficiency of amplification. Fortunately, there are computer programs available that can analyze various factors, as well as many other more subtle ones, thus being well worth the investment.

2.2.2. Primers for RNA Targets

If one wishes to amplify RNA targets, the design of primers becomes somewhat more complex. Four strategies for RNA amplification are possible. The first three techniques begin with destroying the entire genomic DNA with an RNase-free DNase treatment to eliminate all DNA copies of a gene that could lead to false-positive results regarding an RNA target. The DNase is then

inactivated, thus allowing one to convert all mRNA to cDNA with an RT reaction by using one of two types of primers.

The first type of primer is called an oligo d(T) primer. All mRNA molecules are single-stranded and have a poly (A) tail, meaning there is a long series of AAAAAAAAA… at 3'-end of the RNA molecule. An oligo d(T) primer is simply a long series of TTTTTTTs that will anneal to the poly(A) tail. When a reaction is performed with the reverse transcriptase enzyme (RT), the enzyme will extend the primer, making a complementary copy of the mRNA. The RT enzyme synthesizes DNA from the RNA template—substituting thymine for uracil. This reverse transcribed DNA is known as "cDNA." If the reverse transcription reaction is properly carried out, all the mRNA will be converted to the more stable cDNA, which is then available for amplification through conventional PCR techniques.

The second type of primer is called random primers. These are sets of very short oligos of random sequence, generally "hexamers," only six base pairs long. They anneal to complementary strands of mRNA, and the RT enzyme extends them in a manner similar to that described for oligo d(T) primers. Because any hexamer represents a common sequence as the result of its short length and because so many types of hexamers are included in a random primer set, essentially all of the mRNA converted to cDNA by this method (but not necessarily the early parts of every sequence).

Next, one can use a specific primer to reverse-transcribe only the gene-of-interest from the mRNA rather than all mRNA, as with the oligo d(T) and random primers. The cDNA copy can usually be created using the downstream (antisense) primer for the subsequent PCR reaction, resulting in unbounded transcription of the downstream target.

For the last type of RT reaction, please bear in mind that cDNA—which represent copies of mRNA—is fundamentally different from genomic DNA because it represents only the expressed sequences or exons of DNA. Therefore, the cDNA will be missing all of the introns and the controlling regions of DNA that are found in the genomic copies, which in fact compose 70 to 90% of most eukaryotic genomes.

An investigator can exploit this fundamental difference to design special, RNA-specific primers that spans introns in the genomic DNA, eliminating the need for the oligo d(T) primers and the wholesale conversion of mRNA to cDNA (or the alternative downstream specific-primers). Rather, one designs primers that will anneal only to targeted mRNA sequences by designing the primers to span introns in the genomic DNA. The primers will only then adhere to the mRNA templates and the cDNA copies of mRNA, and not to any genomic DNA copy of the same gene.

If one combines these special primers with a special polymerase enzyme that has both RT and DNA polymerase activity, such as the r*Tth* enzyme described

earlier, then one can amplify mRNA sequences directly without going through any specific RT step. This simplifies the whole PCR procedure by eliminating the need for a harsh DNase treatment, as well as a buffer change between the RT and polymerase enzyme steps. Better yet, this procedure allows for the amplification of multiple mRNAs or two types of nucleotide signals simultaneously—both the mRNAs and the genomic DNAs—because there is no need to destroy all the endogenous DNA: primers for each nucleotide-type can also be included without interfering with the activity of the other. However, one must know a considerable amount about the sequence of the gene in question to design these RNA-specific primers.

2.2.3. Length of Desired Amplicon

Recent publications have shown that the amplification of genes up to 50,000 base pairs is possible. However, this "long PCR" is not frequently used for *in situ* work because the primary purpose of the amplification in most circumstances is to detect specific genes, not clone them.

For most *in situ* PCR work, relatively short amplicons are used. Our laboratory has had great success with amplicons in the 150- to 500-bp range, and that is what we usually target. The amplicons should not be so small as to be prone to diffusion away from the original locus of the target, nor should they be so long as to lower the efficiency of the amplification in the difficult environment of a cellular matrix.

2.2.4. Sources for Sequence Data and Computerized Design of Primers

There are several useful sources. First is the scientific literature, particularly if one's project follows earlier research on a similar matter. But be aware: errors in the transcription of tedious DNA sequences seem to be common in the published literature.

A much more useful and up-to-date source for sequence information is GenBank, an extensive, freely available database operated out of the Los Alamos National Laboratory in New Mexico, and accessible on the World Wide Web through the National Library of Medicine. GenBank has sequence data available for a wide variety of genes from various species, though its collection is most extensive with human, primate, and rodent species. The GenBank can be contacted through the Internet or via modem, with sequences downloaded digitally with little or no transcription error (http://www.ncbi.nlm.nih.gov/).

These data are most useful if used in combination with software that is specially designed to process these data and select primer sequences, after various desired characteristics for the primers (or hybridization probes) have been input. One can get free Internet programs to design primers. Also, numerous PCR primer pairs are available as stock items from commercial biotechnology com-

panies. More information on primers, and other products can be obtained via the Internet (i.e., http://www.sciquest.com)

3. Preparation of Tissues

3.1. Cell Suspensions

To use peripheral blood leukocytes, first isolate cells on a Ficoll-Hypaque density gradient. Tissue-culture cells or other single-cell suspensions also can be used.

3.2. Adherent Cultured Cells

There are several types of slides that are designed to support *in situ* PCR after they are attached on the glass slides. The cells are grown on these types of slides. If certain primary cell cultures require attachment factors or growth media, these could be used in conjunction with this cell culture system. After appropriate confluency (usually >60%), cells are gently washed with 1X phosphate-buffered solution (PBS), heat fixed, and then fixed with 2% paraformaldehyde overnight.

3.3. Paraffin-Fixed Tissue

Routinely fixed paraffin tissue sections can be used for amplification purpose quite successfully. This permits the evaluation of individual cells in the tissue for the presence of a specific RNA or DNA sequence. For this purpose, tissue sections are placed on routine histological slides. Tissue sections should be sliced to a 5- to 6-μm thickness. However, if one is using tissues that contain particularly large cells, such as ovarian follicles, then thicker sections may be appropriate. Before *in situ* PCR, the slides need to be deparaffinized.

4. *In Situ* PCR (Basic Preparation, All Protocols)

For all sample types, the following steps comprise the basic preparatory work which must be done before any amplification-hybridization procedure. The overview is depicted in **Fig. 1**.

4.1. Creating Micro-Well for In Situ *PCR*

Before the actual amplification process can be initiated, we have to create a vapor-tight sealing chamber (MJ Research; www.mjr.com/ or www.biorad.com) which can withstand high temperatures required for PCR. Once an artificial well is created, one can place the PCR solution so the putative genes of interest can be amplified *in situ*. For this purpose, we can use "Frame-Seal Incubation Chambers." These frames have double-sided adhesive surfaces: one on the bottom that sticks to the surface of slides, and one on the top that can be sealed by a plastic cover after it has been placed in the correct amount of PCR solution on

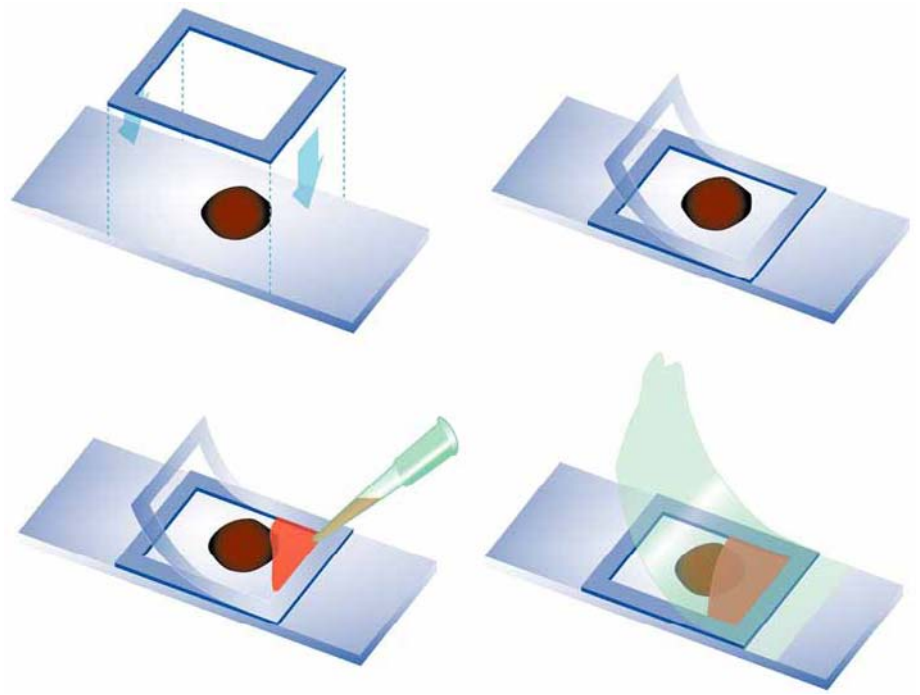

Fig. 1. Overview of *in situ* DNA and RT PCRs.

the tissue surface. To create an artificial well, first the adhesive frame is attached to the slide, enclosing the specimen area. Next, the reaction cocktail is added and the well is sealed with the flexible plastic cover slip. This vapor-tight chamber can withstand temperatures up to 99°C. After the completion of the amplification process, the entire artificially created well can be removed by simply pilling-off the adhesive-frame. In addition, if one requires growing cells or small fragments of tissues inside the artificially created well, then the entire adhesive frame and the slide can be sterilized either by ultraviolet treatment or autoclaving the whole slide with frame before seeding the cell cultures (*see* **Fig. 2** for details).

4.2. Heat Treatment

Place the slides with the adhered tissues or cells on a heat-block at 105°C for 0.5 to 2 min to stabilize the cells or tissue on the glass surface of the slide. This step is absolutely critical. One may need to experiment with different periods in order to optimize the heat treatment for specific tissues. Our laboratory routinely uses 90 s for DNA target sequences, and approx 30 s for the RNA sequences (for RT *in situ* PCR).

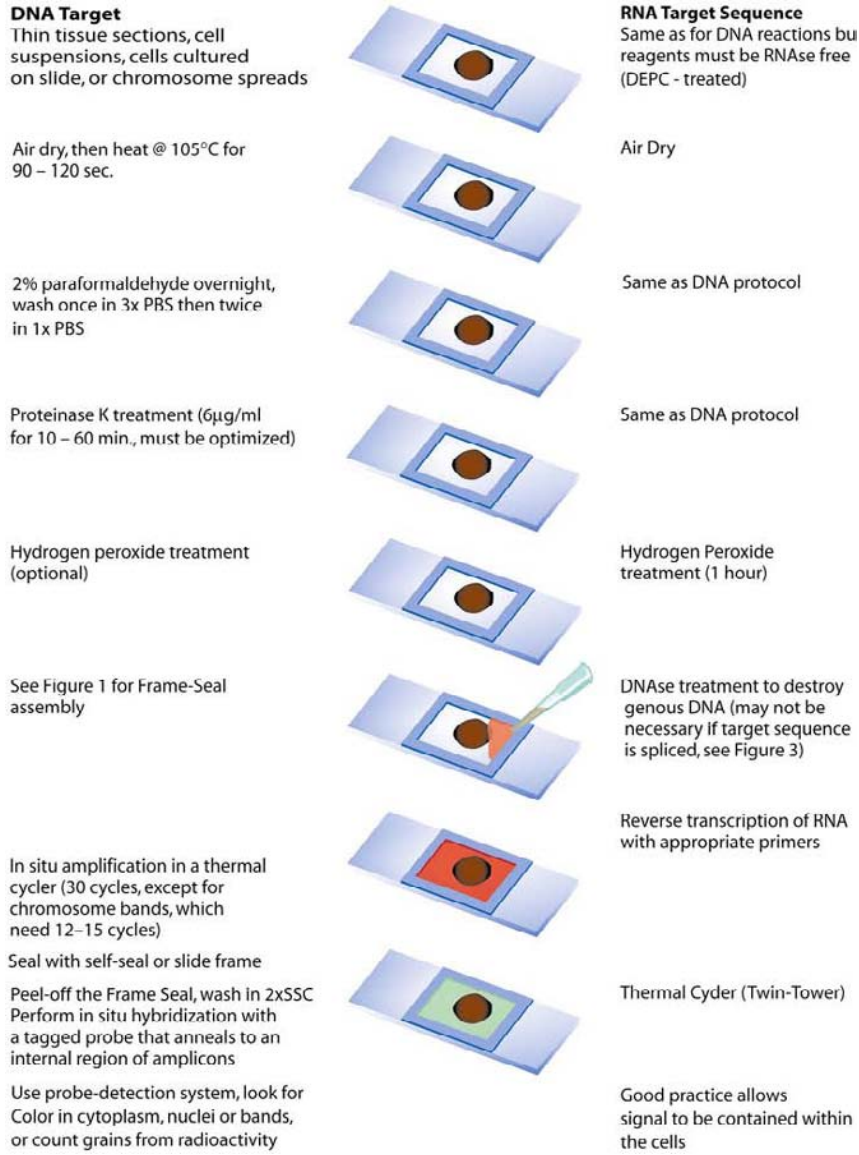

Fig. 2. Overview of frame-seal incubation chambers.

4.3. Fixation and Washes

Place the slides in a solution of 2% paraformaldehyde in PBS (pH 7.4) for 2 h at room temperature. Then, wash the slides once with 3X PBS and 1X PBS for 10 min, agitating periodically with an up and down motion. Repeat once

with fresh 1X PBS. At this point, slides with adhered tissues can be stored at –80°C until use. If biotinylated probes or peroxidase-based color developments are to be used, the samples should further be treated with a 3% solution of hydrogen peroxide in PBS in order to inactivate any endogenous peroxidase activity. Incubate the slides or store at room temperature for 10 to 20 min. If other probes are to be used, proceed directly to the proteinase K digestion, which is perhaps the most critical step in the protocol.

4.4. Proteinase K Treatment

This step is the most critical step in the protocol. Treat samples with 6 µg/mL proteinase K in PBS for 5 to 10 min at room temperature (*see* **ref.** ***20*** for a detailed protocol).

4.5. RT Variation: In Situ *RNA Amplification*

One has two choices in order to detect an RNA signal. The first and more elegant method is to simply use primer pairs that flank spliced sequences of mRNA, as these particular sequences will be found only in RNA and be split into sections in the DNA (*see* **Fig. 3**). Thus, by using these RNA-specific primers, one can skip the following DNase step and proceed directly to reverse transcription. The second, more brutal, yet often necessary approach is to treat the cells or tissue with a DNase solution subsequent to the proteinase K digestion. This step destroys the entire endogenous DNA in the cells so that only RNA survives to provide the signals for amplification.

4.6. RNase and Diethylpyrocarbonate Treatment

All reagents for RT *in situ* amplification should be prepared with RNase-free water (i.e., diethylpyrocarbonate [DEPC]-treated water). In addition, the silanized glass slides and all glassware should be RNase-free, which we insure by autoclaving the glassware in a small autoclave before use in the RT-amplification procedure.

One has to take a great care in performing the RT reaction *in situ*, and there are many caveats that one needs to know before jumping into the action of RT-*in situ* PCR. Usually, a researcher is well-trained in RNA isolation and analysis methods by someone or by technical manuals, which are so abundant. Experimental procedures often are not questioned and quickly become an absolute truth. Furthermore, it is difficult to sort out the “correct” procedure from the literature to document the “facts” from the company line and kits that all claim to be the best in the market. Therefore, we would like to mention certain ground rules that might help the reader in their quest of preserving the RNAs in the cells. One should use DEPC treatment to make solutions RNase-free. However, DEPC may not be the only choice and may not be the only

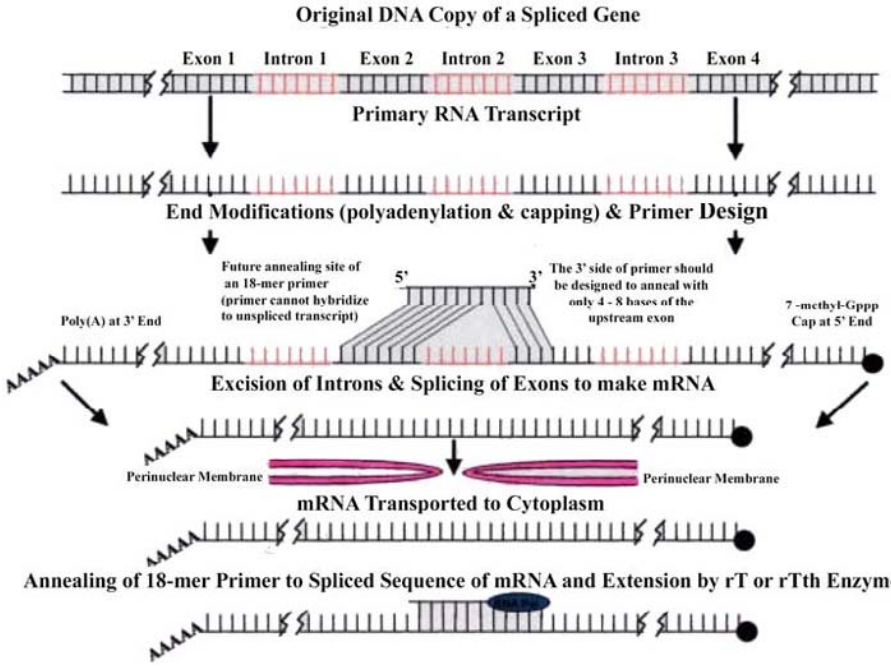

Fig. 3. Overview of multiple splicing system and design for exon-specific primers.

method of getting rid of RNases. In our opinion, one needs to use both autoclaving, as well as DEPC treatment to destroy RNases ***(18,21)***. Many researchers believe that autoclaving is not effective at eliminating RNase in solution, because the RNases simply renature as the solution cools; this is particularly not true. Autoclaving destroys the majority of the RNase contamination. However, if your samples contain a large amount of RNases, than both DEPC and autoclaving would be required ***(21,22)***.

Generally, autoclaving will indeed inactivate a substantial amount of RNase A. For example, in some reports ***(15,23)*** various concentrations of RNase A were added to PBS and autoclaved. Aliquots of each solution were mixed with a 304-base ^{32}P-labeled RNA probe and incubated at 37°C for 1 h, followed by electrophoresis and exposure to film. Without autoclaving, the probe begins to degrade at an RNase concentration of 100 pg/mL. Autoclaving inactivated enough of the RNase A to protect the probe from degradation up to a concentration of 1 µg/mL. Therefore, only a portion of the RNase was inactivated by autoclaving; otherwise, the RNA probe would remain intact at any RNase concentration. Autoclaving alone may be sufficient to eliminate enough RNases for some applications. However, because neither the extent of RNase con-

tamination nor at what RNase concentration the assay is sensitive is known, DEPC should be used to make sure that all of RNases are destroyed.

One should keep in mind that autoclaving does inactivate DEPC by causing hydrolysis of DEPC. CO_2 and ethanol are released as reaction byproducts. DEPC has a half-life of approx 30 min in water, and at a DEPC concentration of 0.1%, solutions autoclaved for 15 min/L can be assumed to be DEPC-free. After autoclaving the DEPC containing solution, a faint ethanol smell may linger after autoclaving, but more commonly a sweet, fruity smell is observed, which is caused by the ethanol byproduct combining with trace carboxylic acid contaminates and forming volatile esters. The smell is not the result of trace DEPC remaining in the solution.

Of note, DEPC does not inactivate RNases in Tris. Tris contains an amino-group which reacts with DEPC and makes it unavailable to inactivate RNase ***(19)***. Tris and HEPES do indeed make DEPC unavailable to inactivate RNase at a DEPC concentration of 0.1% (recommended by most protocols). However, 1% DEPC is sufficient to overcome this effect. However, one has to be concious, as high levels of residual DEPC or DEPC byproducts in a solution can inhibit some enzymatic reactions or chemically alter (carboxymethylate) RNA. It has been documented that DEPC byproducts in RNA samples can inhibit in vitro translation reactions ***(15–19)***. It would be difficult to predict the different interactions of DEPC with various molecular biology reagents that are being used in a RT-reaction *in situ*. The most cautious approach for making RNase-free solutions would be to mix molecular biology-grade powdered reagents in DEPC-treated water. Alternatively, many premade nuclease-free solutions can be purchased from many different commercial sources.

4.7. RT Reaction

Next, one wishes to make cDNA of the targeted RNA sequence so that the signal can be amplified. One should follow the manufacturer's suggestions to prepare the cocktail. After preparing the RT-PCR solution, one should follow the protocol described in **Subheading 3.4.2.**

4.8. RT-Primers and Target Sequences

In our laboratory, we simply use antisense downstream primers for our gene of interest, as we already know the sequence of most genes we study. However, one can alternatively use oligo (dT) primers to first convert all mRNA populations into cDNA, and then perform the *in situ* amplification for a specific cDNA. This technique may be useful when performing amplification of several different gene transcripts at the same time in a single cell. For example, if one is attempting to detect various cytokine expressions, one can use an oligo (dT) primer to reverse transcribe all of the mRNA copies in a cell or tissue section.

Then, one can amplify more than one type of cytokine and detect the various types with different colored probes that develop into different colors.

In all RT reactions, it is advantageous to reverse-transcribe only relatively small fragments of mRNA (<1500 bp). Larger fragments may not completely reverse-transcribe because of the presence of secondary structures. Furthermore, the RT enzymes—AMVRT and MMLVRT, at least—are not very efficient in transcribing large mRNA fragments. However, there are several second-generation RT enzymes now available that are more efficient then their predecessors.

4.9. Thermal Cyclers

Various technologies of thermocyclers will work in this application; however, some instruments work much better than others. We use dedicated slide thermocyclers that are specifically designed to hold 16 or 32 slides. We understand that other labs have used stirred-air, oven-type thermocyclers quite successfully; however, we have also heard that there are sometimes problems with the cracking of glass slides during cycling. Thermocyclers dedicated to glass slides are now available from several vendors, including Barnstead Thermolyne of Iowa, Coy Corporation of Minnesota, Hybaid of England, Perkin Elmer of California, and MJ Research of Massachusetts. Our laboratory has used an MJ Research PTC-100-16MS, DNA-Engine Twin-Tower 16 × 2 quite successfully. Recently, this company has combined the slide and tubes into a single block, allowing the simultaneous confirmation of *in situ* amplification in a tube. Furthermore, there are newer designs of a thermal cycler that incorporate humidification chambers as well temperature gradient to optimize the annealing temperatures for PCR. The gradient thermocyclers are especially useful in the optimization of annealing steps, reverse transcription, and hybridization steps.

We suggest that you follow the manufacturer's instructions on the use of your own thermocycler, bearing in mind the following points: (1) Glass does not easily make good thermal contact with the surface on which it rests. Therefore, a weight to press down the slides and/or a thin layer of mineral oil to fill in the interstices will help thermal conduction. If using mineral oil, make certain that the oil is well smeared over the glass surface so that the slide is not merely floating on air bubbles beneath it; (2) the top surfaces of slides lose heat quite rapidly through radiation and convection; therefore, use a thermocycler which envelopes the slide in an enclosed chamber (as in some dedicated instruments), or insulate the tops of the slides in some manner. Insulation is particularly critical when using a weight on top of the slides, for the weight can serve as an unwanted heat sink if it is in direct contact with the slides; and (3) good thermal uniformity is imperative for good results—poor uniformity or irregular thermal change can result in cracked slides, uneven amplification, or completely

failed reactions. If adapting a thermocycler that normally holds plastic tubes, use a layer of aluminum foil to spread out the heat.

4.10. Direct Incorporation of Nonradioactive Labeled Nucleotides

Several non-radioactive labeled nucleotides are available from various sources (i.e. dCTP-Biotin, digoxin II-dUTP, etc.). These nucleotides can be used to directly label amplification products; then, the proper secondary agents and chromogens can be used to detect the directly labeled *in situ* amplification products. However, the greatest specificity is only achieved by conducting amplification followed by subsequent *in situ* hybridization. In the direct labeling protocols, nonspecific incorporation can be significant, and even if this incorporation is minor, it still leads to false-positive signals similar to nonspecific bands in gel electrophoresis following solution-based DNA- or RT-amplification. In case of a solution-based PCR, one generally does not notice the nonspecific amplification bands if it is less than 0.2 µg. However, in the case of *in situ* PCR, in which one is working at the single-cell level, a minutely amplified signal can be observed easily under a high magnification microscope. Therefore, we strongly discourage the direct incorporation of labeled nucleotides as part of an *in situ* amplification protocol.

4.11. Multiple Signals, Multiple Labels in Individual Cells

DNA, mRNA, and protein can all be detected simultaneously in individual cells ***(12,13)***. One can label proteins by rhodamine-labeled antibodies. Then, one can perform both RNA and DNA *in situ* amplification in the cells. If one is using primers for spliced mRNA and if these primers are not going to bind any sequences in DNA, then both DNA and RT amplification can be conducted simultaneously. Of course one still needs to perform the RT-step, but this time without pre-DNase treatment. Subsequently, products can be labeled with different kinds of probes, resulting in different colors of signal. For example, proteins can have a rhodamine-labeled probe, mRNA can show a fluorescein isothiocyanate signal (fluorescein isothiocyanate-conjugated probe, more than 20 different fluorochromes are available) and DNA can be labeled with a biotin-peroxidase probe or a fluorochrome with different color emission. Each will show a different signal within an individual cell ***(12,13)***.

5. Hybridization

The *in situ* hybridization (ISH) technique has been successfully applied in both the research and clinical settings. However, one single, easy-to-use universal procedure has not been developed. Therefore, the specific needs of the diagnostic or research goals must be considered in choosing a suitable protocol. We will leave this up to each of the investigators to determine their opti-

mal protocol. Following are some general basic information that needs to be kept in mind.

The most successful procedure would be where an investigator has optimized the ISH of a specific probe with the target genes (in this case the amplicons in the cells of interest) while minimizing background signals (reviewed in ***[15]***). It is the critical factor for ISH sensitivity. Background signals arise primarily from nonspecific retention of a probe in tissue sections (resulting from electrostatic interactions between probe and tissue macromolecules) and entrapment of probes in the spherical lattice of the tissue section ***(24–30)***. Several chemically functional groups in proteins (i.e., carboxylate and amine groups) may be responsible for this nonspecific binding. There are several protocols to minimize the background signals by treating tissue slides with acetic anhydride and triethanolamine ***(27)***. Acetylation of amine groups by acetic anhydride, routinely used in ISH protocols, maybe important in reducing backgrounds for probes larger than 2.0 kb ***(29)***.

Another way to decrease nonspecific probe binding is to saturate the binding sites on proteins by incubating tissues with prehybridization solution, which typically includes Ficoll, bovine serum albumin, polyvinyl pyrrolidone, and nucleic acids. These reagents also are present in hybridization buffers to compete with the nonspecific binding of probes to tissue. However, this is not a fool-proof method, and it does not completely prevent background signals. Nuclease treatment after hybridization is still necessary for reducing this nonspecific signal (nuclease treatment degrades unhybridized, single-stranded probes). Some investigators have found that without RNase treatment, the background with [^{33}P]-labeled RNA probes was so high that specific hybridization signals was not discernable. Even high stringency washing did not remove this background. Generally, RNA probes tend to exhibit high levels of nonspecific binding, so RNase treatment must be applied in ISH studies when RNA probes are used ***(30)***.

Antisense RNA probes are used widely for ISH because they have been demonstrated to be more specific and sensitive than cDNA probes or oligoprobes ***(15,24)***. Both isotopically and nonisotopically labeled probes have been used successfully for ISH. ^{33}P and ^{35}S frequently are used isotopes to label probes. ^{35}S-labeled RNA probes usually give higher backgrounds, so when ^{35}S-labeled probes are used, dithiothreitol should be added to all solutions used in prehybridization, hybridization, and posthybridization washes. If one is going to use radioisotope labeled probes, then we recommend using ^{33}P-labeled probes for ISH studies because they result in lower background and higher resolution as compared with ^{35}S-labeled RNA probes.

Nonisotopic labeled probes (i.e., biotin, or chemiluminescent labeled) are the most frequently used for ISH studies. The sentitivity of these probes may be

significantly lower than the radioisotopic probes but for post-*in situ* PCR, they are the ideal probes to be used.

Although there are different recipes for making hybridization buffers, the inclusion of dextran sulfate in the hybridization solution increases probe binding to target mRNA, that is, including 10% dextran sulfate enhances ISH signal several fold. However, too much dextran sulfate in the hybridization buffer will induce high background, which is difficult to remove in posthybridization washes. Various methods to be used for different kinds of applications have been described in detail in several of our previous publication ***(15,20,31)***.

6. Controls

The validity of *in situ* amplification–hybridization should be examined in every run. Attention here is especially necessary in laboratories first using the technique because occasional technical pitfalls lie on the path to mastery. In an experienced laboratory, it is still necessary to continuously validate the procedure and to confirm the efficiency of amplification. To do this, we routinely run two or three sets of experiments in multiwelled slides simultaneously, for we must not only validate amplification, but we must also confirm the subsequent hybridization/detection steps.

In our laboratory, we frequently work with infectious agents. A common validation procedure we conduct is to mix infected cells with uninfected cells in known ratios (i.e., 1:10, 1:100, etc.), and then confirm that the results are appropriately proportionate. To examine the efficiency of amplification, one can use a cell line, which carries a single copy, or two copies of the gene of interest ***(1,13,32–35)***, and then look to see that proper amplification and hybridization has occurred.

In all amplification procedures, we use one slide as a control for nonspecific binding of the probe. Here we hybridize the amplified cells with an unrelated probe. We also use human leukocyte antigen-DQ and β-actin probes and primers with human peripheral blood mononuclear cells and other tissue sections as positive controls to check various parameters of our system.

In case one is using tissue sections, a cell suspension lacking the gene of interest can be used as a control. These cells can be added on top of the tissue section and then retrieved after the amplification procedure. The cell suspension can then be analyzed with the specific probe to see if the signal from the tissue leaked out and entered the cells floating above.

We suggest that researchers carefully design and use appropriate positive and negative controls for their specific experiments. In the case of RT–*in situ* amplification, one can use β-actin, γ-globulin, human leukocyte antigen-DQa, and other endogenous-abundant RNAs as the positive markers. Of course, one should always have an RT-negative control for RT *in situ* amplification, as well

as DNase and non-DNase controls. Controls without polymerase plus primers and without primers should always be included.

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Index