

PRINS Combined With Peptide Nucleic Acid Labeling

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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-

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 (Interger Company, New York, NY), stored at 4°C.
5. Water bath at 73°C.
6. Light microscope Leica DMLB with $\times 10$ and $\times 40$ magnification (Leica France, Rueil-Malmaison, France).

2.2. PRINS Reaction

1. 2'-Deoxyadenosine 5'-triphosphate (dATP): 100 mM solution (Roche Diagnostics, Meylan, France) diluted 1:10 with sterile distilled H₂O.
2. 2'-Deoxycytosine 5'-triphosphate (dCTP): 100 mM solution (Roche Diagnostics) diluted 1:10 with sterile distilled H₂O.
3. 2'-Deoxyguanosine 5'-triphosphate (dGTP): 100 mM solution (Roche Diagnostics) diluted 1:10 with sterile distilled H₂O.
4. 2'-deoxythymidine 5'-triphosphate (dTTP): 100 mM solution (Roche Diagnostics) diluted 1:100 with sterile distilled H₂O.
5. Fluorescein-12-2'-deoxyuridine 5'-triphosphate (FITC-12-dUTP), 1 mM (Roche Diagnostics).

6. Bovine serum albumin (BSA; Sigma, St. Louis, MO).
7. *Taq* DNA polymerase (Roche Diagnostics) or *AmpliTaq* (Perkin Elmer, Foster City, CA).
8. 10X *Taq* buffer: 500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM MgCl₂.
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 diethylaminocoumarine. 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, Altussheim, 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 μ L containing: 0.2 mM of dATP, dCTP, and dGTP; 0.02 mM of dTTP; 0.02 mM of FITC-12-dUTP; 50mM KCl, 10 mM Tris-HCl, pH 8.3; 1.5 mM $MgCl_2$; 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 μ L of each 1:10 diluted dATP, dCTP and dGTP; 1 μ L of the 1:100 diluted dTTP; 1 μ L of FITC-12- dUTP; 1 μ L of BSA; 5 μ L of 10X *Taq* buffer, 0.5 μ L of the *Taq* DNA polymerase, 4 μ L of the primer specific for chromosome 9, and distilled water to 50 μ L.
2. Place the reaction mixture under a 22 \times 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 \times 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 $\mu\text{L}/\text{mL}$) and DAPI (0.3 $\mu\text{L}/\text{mL}$). Use 15 to 20 μL of mountant/slide.
3. Cover with a 22 \times 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

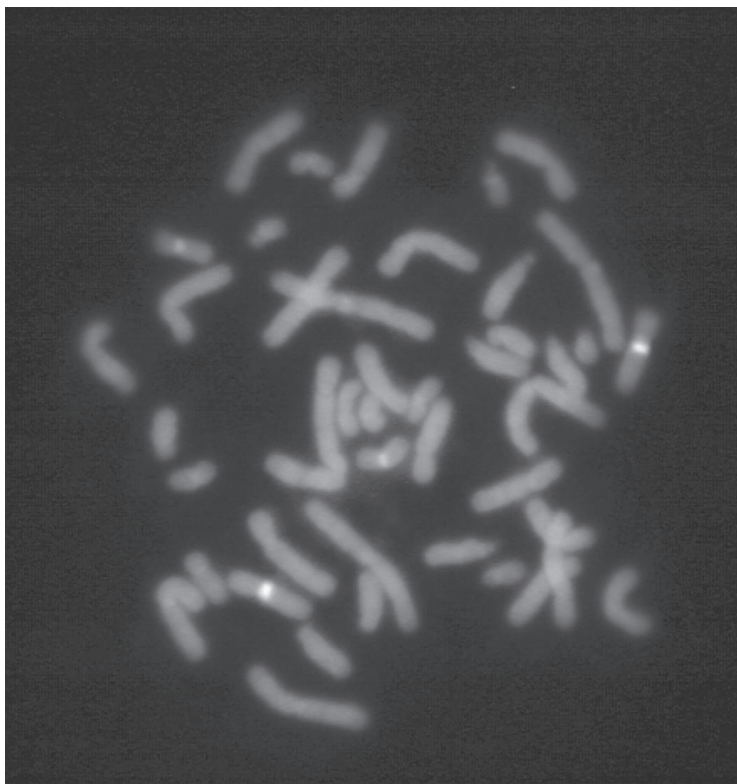


Fig. 1. Combined PRINS and PNA labeling on a chromosome spread. Chromosome 9 is labeled by PRINS in green. Chromosomes 1 and 18 are labeled by PNA probes, in blue and red, respectively. (Please *see* color insert following p. 48.)

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, Denmark), 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.

Acknowledgments

The experiments performed for elaborating this protocol were supported by the Projet Hospitalier de Recherche Clinique, a French research project (no. 7732), from the Centre Hospitalo-Universitaire of Montpellier and a European INTAS project no. 03-51-4060. P. Paulasova was supported by a Czech fellowship VZ FNM 00000064203

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<http://www.springer.com/978-1-58829-549-1>

PRINS and In Situ PCR Protocols

Pellestor, F. (Ed.)

2006, XVI, 244 p., Hardcover

ISBN: 978-1-58829-549-1

A product of Humana Press