PRINS DNA Synthesis on Frozen Tissue Sections

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1. Introduction

Primed in situ (PRINS) labeling has become an alternative to in situ hybridization (ISH) for the localization of nucleic acid sequences in cell preparations (1–4). In the PRINS method, an unlabeled primer (restriction fragment, PCR product, or oligonucleotide) is annealed to its complementary target sequence in situ. The primer serves as an initiation site for in situ chain elongation using a thermostable DNA polymerase and labeled nucleotides, which can be detected directly by fluorescence microscopy, such as fluorochrome-labeled dNTPs, or indirectly using, e.g., biotin- or digoxigenin-dUTP and the application of fluorochrome-conjugated avidin or antibody molecules (3,5,6). The detection limit of the PRINS technique appears to be in the order of low-copy sequences (3,7).

Here we describe an easy protocol for the application of the PRINS labeling reaction to frozen tissue sections (8). In this way, individual cells can be identified in their tissue context and be analyzed for their copy numbers of specific chromosome regions. Both tissue fixation and proteolytic digestion before performing the PRINS reaction proved to be the critical steps in the total procedure permitting access of the PRINS reactants, while preserving the morphology of the nuclei in the tissue. Such pretreatment steps have also been shown to be essential for efficient application of ISH to tissue sections (9,10). Examples are shown of fluorescence and bright-field detection protocols for labeled DNA sequences in normal diploid tissue nuclei (Fig. 1C–E).
Fig. 1. (see color plate number 2 after p. 82) (C) Bright-field detection of chromosome 9 centromeres with biotin/PO-TMB in a frozen tissue section of normal colon epithelium after PRINS, hematoxylin counterstaining, and immersion oil embedding. (D) Bright-field detection of chromosome 9 centromeres with biotin/PO-DAB in a frozen tissue section of normal bladder epithelium after PRINS, hematoxylin counterstaining, and PBS/glycerol (1:9) embedding. (E) Direct fluorescence detection of chromosome 9 centromeres with fluorored (red) in a frozen tissue section of normal bladder epithelium after PRINS and PBS/glycerol/DABCO embedding with DAPI counterstaining.

2. Materials

2.1. PRINS DNA Labeling

1. Pepsin from porcine stomach mucosa (2500–3500 U/mg) (Sigma, St. Louis, MO).
2. Ultrapure dNTP set (Pharmacia, Uppsala, Sweden): 100-mM solutions of dATP, dCTP, dGTP, and dTTP.
3. Biotin-16-dUTP, digoxigenin-11-dUTP, fluorescein-12-dUTP (Boehringer, Mannheim, Germany), fluorored-dUTP (Amersham, Little Chalfont, UK).
4. Oligonucleotide primers (see Table 1 of Chapter 3) at 250 ng/µL.
5. Taq DNA polymerase (Boehringer) or AmpliTaq (Perkin Elmer, Chalfont St. Giles, UK).
6. Bovine serum albumin (BSA) (Sigma).
7. 20X SSC: 3M NaCl, 300 mM trisodium citrate, pH 7.0.
8. 10X Tag buffer: 500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM MgCl₂, 0.1% BSA.
9. PRINS stop buffer: 500 mM NaCl, 50 mM EDTA, pH 8.0.
10. Washing buffer: 4X SSC (diluted from 20X SSC), 0.05% Triton X-100.
11. Ethanol/37% HCl (100:1)-cleaned microscope slides and coverslips.
12. Rubber cement.
13. Water bath at 65°C.
14. Thermal cycler (Hybaid Omnigene Flatbed) (Hybaid, Teddington, UK).

2.2. Cytochemical Detection

1. Dried skimmed milk powder.
2. Normal goat serum (NGS).
3. Horseradish peroxidase-conjugated avidin (AvPO) (Dako, Glostrup, Denmark).
4. Horseradish peroxidase-conjugated sheep antidigoxigenin Fab fragments (SHADigPO) (Boehringer).
5. 30% H₂O₂ (Merck, Darmstadt, Germany).
6. Diaminobenzidine (DAB) (Sigma).
7. 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma).
8. Diocetyl sodium sulfosuccinate (DSSS) (Sigma).
9. Sodium tungstate (Sigma).
10. Immersion oil (Zeiss).
12. 4′,6-Diamidino-2-phenyl indole (DAPI) (Sigma).
13. PO-DAB buffer: 0.1M imidazole (Merck) in PBS, pH 7.6.
14. PO-TMB buffer: 100 mM citrate-phosphate buffer, pH 5.1.
15. Hematoxylin: Hematoxylin (Solution Gill no. 3) (Sigma); distilled water (1:4).
16. Blocking buffer: 4X SSC (diluted from stock 20X SSC), 0.05% Triton X-100, 5% skimmed milk powder.
17. Washing buffer: 4X SSC, 0.05% Triton X-100.
18. Incubator at 37°C.
20. Kodak 400 ASA and 100 ASA film.

3. Methods

3.1. PRINS DNA Synthesis

1. Fresh tissue samples obtained after surgical resection are snap frozen in liquid nitrogen. From each sample, cut 4-μm sections with a cryostat, mount them on poly-L-lysine-coated slides, and store at −20°C until use.
2. Air-dry slides, fix in methanol:acetic acid (3:1) for 10 min at room temperature (see Note 1), and air-dry again.
3. Wash slides for 5 min in PBS and 2 min in 0.01M HCl.
4. Treat samples with 100 μg/mL pepsin in 0.01M HCl for 10 min at 37°C, wash for 2 min in 0.01M HCl at 37°C, and pass the slides through an ethanol series starting with 70% ethanol in 0.01M HCl (Note 2).
5. Postfix samples in 1% paraformaldehyde in PBS for 20 min at 4°C, wash in PBS for 5 min, dehydrate, and subject slides to the PRINS procedure.

6. Prepare the PRINS reaction mix on ice as follows: Dilute 100 mM dATP, dGTP, and dCTP 1:10 with distilled water. Dilute 100 mM dTTP 1:100. Put together in a microcentrifuge tube: 1 μL of each of the diluted dNTPs, 1 μL of either 1 mM biotin-16-dUTP, digoxigenin-11-dUTP, fluorescein-12-dUTP, or fluorored-dUTP (see Note 3), 5 μL of 10X Taq buffer, 250 ng of oligonucleotide (see Note 4), 1 U Taq polymerase, and distilled water to 50 μL.

7. Place 40 μL of this mixture under a coverslip on the slide, seal with rubber cement, air-dry the rubber cement, and transfer to the heating block of the thermal cycler.

8. Each PRINS reaction cycle consists of 2 min at 94°C (for denaturation of cellular DNA, see Note 5), 5 min at the appropriate annealing temperature (see Note 6), and 15 min at 72°C for in situ primer extension.

9. Stop the PRINS reaction by transferring the slides (after removal of the rubber solution seal) to 50 mL of PRINS storage buffer in a Coplin jar at 65°C for 1 min.

10. Transfer the slides to washing buffer at room temperature, and wash for 5 min.

3.2. Enzyme Cytochemical Detection

1. Place 40 μL of blocking buffer under a coverslip on the slide, and leave for 5 min at room temperature to reduce background staining in the detection procedures.

2. Wash slides for 5 min in washing buffer.

3. For reactions using biotin-16-dUTP: Dilute AvPO 1:100 in blocking buffer and apply 50 μL under a coverslip. Incubate slides for 30 min at 37°C in a humid chamber (Note 7).

4. For reactions using digoxigenin-11-dUTP: Dilute SHADigPO 1:100 in blocking buffer and treat as in step 3 (Note 7).

5. Fluorescein-12-dUTP and fluorored-dUTP need no additional reporter and are simply mounted as described in step 11 (see Note 7).

6. Wash slides for 2 × 5 min in washing buffer and for 5 min in PBS.

7. Visualize the PRINS-labeled DNA target by an appropriate horseradish peroxidase (PO) reaction (11; see also Chapter 3):
   a. Horseradish peroxidase-diaminobenzidine (PO-DAB) reaction: Mix 1 mL 5 mg/mL DAB in PBS, 9 mL PO-DAB buffer, and 10 μL 30% H₂O₂ just before use, and overlay each sample with 100 μL under a coverslip. Incubate the slides for 5–15 min at 37°C, wash 3 × 5 min with PBS, and dehydrate optionally.

   b. Horseradish peroxidase-tetramethylbenzidine (PO-TMB) reaction: Dissolve 100 mg sodium tungstate in 7.5 mL PO-TMB buffer, and adjust the pH of this solution to 5.0–5.5 with 37% HCl. Just before use, dissolve 20 mg DSSS and 6 mg TMB in 2.5 mL 100% ethanol at 80°C. Mix both solutions with 10 μL H₂O₂, and overlay each sample with 100 μL under a coverslip. Incubate the slides for 1–2 min at 37°C, wash 3 × 1 min with ice-cold 0.1 M phosphate buffer (pH 6.0), and dehydrate.
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8 After performing the enzyme reaction, counterstain the samples with hematoxylin, wash for 5 min in tap water and 2 min in distilled water, and air-dry if you wish.
9. Mount samples with the PO-DAB or PO-TMB precipitate in an organic mounting medium or immersion oil. The PO-DAB precipitate can also be embedded in an aqueous mounting medium. Mount samples with fluorescent PRINS signals in Vectashield containing 0.5 μg/mL DAPI.
10. Examine slides with absorption PRINS signals under a bright-field microscope. Microphotographs can be made using blue and magenta filters and Kodak 100 ASA film. Slides with fluorescent PRINS signals can be analyzed under a fluorescence microscope equipped with suitable filters. Selected cells can be either directly photographed using Kodak 400 ASA film, visualized with a charge-coupled device (CCD) camera, or scanned with a confocal scanning laser microscope (CSLM).

4. Notes

1. Fixation of frozen tissue sections with other fixatives, such as acetone (10 min at −20°C), methanol (10 min at −20°C), methanol/acetic acid (1 min at −20°C/3 × 5 s room temperature), 70% ethanol (10 min at −20°C), and 70% ethanol/1% formaldehyde (10 min at −20°C), resulted in poor preservation of cell morphology after PRINS. In addition, we frequently observed fluorescent staining of the entire nucleus after PRINS labeling of methanol/acetone-fixed tissue sections, probably caused by nuclease activities that survived methanol-acetone fixation (8).
2. Dehydration of the samples after pepsin treatment starting with 70% ethanol in 0.01M HCl helps in preserving cell morphology (manuscript in preparation).
3. In the case of labeling with biotin-16-dUTP or fluorescein-12-dUTP, a four times decrease of the concentration of dTTP in the PRINS reaction mix resulted in significant stronger labeling of DNA sequences. Under the described standard conditions, digoxigenin-11-dUTP and fluorored-dUTP provide the highest sensitivity. However, all the modified nucleotides are suitable for detection of repeated sequences in situ.
4. The concentration of the appropriate oligonucleotide resulting in positive signals needs to be determined by experiment. Generally, 250 ng/slide in 40 μL is used for primers of 16–30 bases complementary to repeated sequences.
5. Separate denaturation of cellular DNA in 70% formamide/2X SSC, pH 7.0, for 2 min at 70°C before the PRINS reaction, as is usually performed for chromosome preparations, resulted in no or only weak PRINS labeling of DNA sequences in situ. Whether this is caused by inefficient primer annealing or extension is not clear at the moment. The same phenomenon is also observed for PRINS on ethanol-fixed cells (see Chapter 8).
6. The optimum primer annealing temperature is only determined empirically. We usually try a series from 45 to 70°C, in 5°C steps.
7. Amplification of PRINS signals can be achieved as follows:
   a. AVPO detection of biotin-16-dUTP may be followed by incubation with biotinylated goat antiavidin (Vector), 1:100 diluted in blocking buffer, and again AVPO.
b. SHADigPO detection of digoxigenin-11-dUTP may be substituted with incubation with monoclonal mouse antidigoxin (Sigma), PO-conjugated rabbit antimouse IgG (Dako), and PO-conjugated swine antirabbit IgG (Dako).

c. FITC-12-dUTP signals may be amplified by incubation with monoclonal mouse anti-FITC (Dako) and FITC-conjugated rabbit antimouse IgG (Dako).

d. Fluorored-dUTP signals cannot be amplified, since antirhodamin antibodies are not commercially available at this time.

e. Amplification of PRINS signals may also be achieved by combining these detection systems with peroxidase-mediated deposition of hapten- or fluorochrome-labeled tyramides (12, 13).

References


