

Protocol of PNA Telomere probe for FISH (Fluorescence *in situ* hybridization)

Introduction

Telomeres have important functions in the stability and replication of chromosomes. These functions are mediated by highly conserved repeats which consist of (TTAGGG)_n in all vertebrates. The number of telomeric repeats in human somatic cells appears to decrease with cell divisions and with age. Telomere shortening may act as a mitotic clock in normal somatic cells and high levels of the enzyme telomerase (capable of elongating telomeres) have been found in tumor cells. The most commonly used tool to estimate telomere length is southern analysis of genomic DNA digested with selected restriction enzymes. Such analysis requires thousands of cells and provides only a crude estimate of the average number of TTAGGG repeats in the chromosomes of all cells analyzed. In principle, fluorescence *in situ* hybridization (FISH) should be able to provide information on the telomere length of individual chromosomes. Directly labeled oligonucleotide probes are attractive probes for such analysis because of their small size (good penetration properties), single strand nature (no denaturation of probe) and controlled synthesis. However the efficiency of oligonucleotide hybridizations for telomeric repeats has not been sufficient to extend this approach beyond qualitative studies of TTAGGG repeat sequences in chromosomes of various species. Recently, it was shown that peptide nucleic acid (PNA) oligonucleotide probes will hybridize with complementary oligonucleotide sequences and that the resulting duplexes are more stable than DNA/DNA or DNA/RNA duplexes. In PNA, the charged phosphate-(deoxy) ribose backbone of conventional DNA and RNA oligonucleotides is replaced by an uncharged backbone of repeating N-(2-amino ethyl)-glycine units linked by peptide bonds. In comparison with DNA oligonucleotides, PNA oligomers demonstrate the higher sequence specificity, improved stability, reproducibility, and lower background. Due to the higher T_m of PNA/DNA duplexes, short (18-mer) Telomere PNA (CCCTAA)₃ are widely used.

Things to do before starting

I. Prepare the Telomere PNA

1. Centrifuge the tubes before opening them in order to collect lyophilized PNA at the bottom of the tubes.
2. Add distilled water to each tube to obtain an approximately $67 \mu\text{g/ml}$ stock solution of each PNA.

PNA	Distilled water	Final concentration
5 nmol	0.5 ml	$67 \mu\text{g/ml}$
10 nmol	1 ml	$67 \mu\text{g/ml}$

3. Dilute telomere PNA probe in PNA hybridization buffer to a final concentration of 800 ng/ml
4. Place $15 \mu\text{l}$ of diluted PNA probe onto the specimen.

✓ **Note:** Store the Telomere PNA probe solution in the dark at $4 \text{ }^\circ\text{C}$.

II. Prepare Solutions

1. Hybridization buffer

10 mM NaHPO_4 , pH 7.4
10 mM NaCl
20 mM Tris, pH 7.5
70% formamide

2. Fixative solution

Prepare Methanol : Glacial acetic acid (3:1) solution.

✓ **Note:** Prepare just before use!

3. Pepsin 0.005% solution

2.5 ml of 10% Pepsin stock in 50 ml 0.01 M HCl.

✓ **Note:** Make fresh! Warm to $45 \text{ }^\circ\text{C}$ before use.

4. Washing solution

- Washing Sol'n I : PBS/0.1% Tween-20
- Washing Sol'n II : 2 × SSC/0.1% Tween-20

III. Equipment

- Diamond pen
- CO₂ incubator
- Centrifuge
- Slide glass
- Coverslips, 22 mm × 22 mm, 0.15 mm
- Humidifier chamber
- Fluorescence microscope with DAPI filter set and filter set appropriate for the PNA
Telomere probe you purchase (Cy3 or FITC or FAM)

IV. Reagents

- Heparin or EDTA
- Phytohaemagglutinin (PHA-P) (-20 °C)
- Colcemide (4 °C)
- PBS (Phosphate Buffered Saline)
- Potassium chloride
- Formaldehyde
- Ethanol (70%, 85% and 100%)
- Pepsin
- DAPI solution
- Mounting media

Procedure

I. Peripheral blood mononuclear cell (PBMC) Culture for Chromosome Analysis

1. Set up a 50 ml culture containing: 39 ml culture medium, 7.5 ml Fetal Bovine Serum, 0.5 ml Phytohaemagglutinin (PHA-M), 0.5 ml 100× penicillin/streptomycin, 2.5 ml Blood (with EDTA or heparin)

✓ **Note:** Phytohaemagglutinin (PHA-M) is used for the stimulation of lymphocyte mitogenic. Both EDTA and heparin prevent the coagulation of blood.

2. Incubate for 72 hours in a CO₂ incubator.

3. Add 500 μl (10 μg/ml) colcemid to 50 ml cell culture.

✓ **Note:** The colcemid depolymerises microtubules and limits microtubule formation (inactivates spindle fiber formation), thus arresting cells in metaphase and allowing cell harvest and karyotyping to be performed.

4. Incubate for 1~2 hours at 37 °C.

5. Harvest cells by centrifugation at 1500 rpm for 5 minutes.

6. Remove the supernatants.

7. Add 30 ml PBS and resuspend cells.

8. Harvest cells by centrifugation at 1500 rpm for 5 minutes.

9. Add 30 ml 75 mM KCl to sample, resuspend, and incubate at room temperature for 30 min.

✓ **Note:** Discard the KCl solution after a month.

10. Add 2 ml freshly made fixative solution and mix carefully by turning the tube.

✓ **Note:** Fixative solution should be used as cold state.

11. Repeat step 10.

12. Harvest cells by centrifugation at 1500 rpm for 5 minutes.

13. Remove supernatant.

14. Add 10 ml fixative solution and mix carefully by turning the tube.

15. Harvest cells by centrifugation at 1500 rpm for 5 minutes.

16. Remove the supernatants.

17. Repeat step 14-16 ($\times 4$)

✓ **Note:** If the nuclei appear to be surrounded by any trace of cytoplasm when the slides are viewed under phase contrast, repeat the fixation step in the protocol (steps 14-16). Cytoplasm interferes with the penetration of the probes leading to problems with hybridization and increased background signal.

18. Add 700 μl fresh fixative solution until a milky suspension of cells is formed without clumps.

19. Clean slides by dipping in 100% ethanol, then dipping in fixative solution dry.

✓ **Note:** Improperly cleaned slides can interfere with hybridization of the PNA probe to the target.

20. Dry slides on air

21. A drop of the cell suspension onto a clean slide using a pipette.

22. Incubator for 10 minutes at 67 ± 2 °C.

II. Hybridization

1. Immerse the slide in PBS for 15 min.

2. Fix the slide in 4% formaldehyde in PBS for 4 min.

3. Wash in PBS for 5 min ($\times 2$).

4. Immerse the slide in 0.005% Pepsin for 4 min at 37°C.

5. Wash in PBS for 3 min ($\times 2$).

6. Dehydrate slides in cold ethanol series (for 1 min in 70%, 85%, 100%).

7. Dry slides on air.

8. To each slide add 15 μl telomere PNA probe in Hybridization Buffer.
9. Cover slides with a coverslip.
10. Denature slides for 5 min at 80°C.
- ✓ **Note:** Denaturation should be performed between at minimum 80°C and maximum 90°C.
Check the temperature of the incubator carefully.
Denaturing temperature below 75°C impairs result seriously.
11. Leave slides for 30 min~2 hrs at room temperature (light protected).

III. Washing

1. Immerse the slide in Washing Sol'n I at room temperature to remove the coverslips.
2. Wash the slide in Washing Sol'n I for 20 min at 57°C.
3. Wash the slide in Washing Sol'n II for 1 min at room temperature.

IV. Counterstaining

1. Stain the slide for 20 minutes in the DAPI solution.
2. Wash the slide in Washing Sol'n II.
3. A drop of mounting media to the target area of the slide.
4. Cover with a coverslip and allow the solution to spread evenly under the coverslip.
Avoid air bubbles.
5. Observe the stained slide using an fluorescence microscope with the appropriate filters.

V. References

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