Telomeric PNA FISH on Metaphase Chromosomes

Required Solutions/Reagents

Blocking reagent (Roche 11096176001)
Stock is 10%, dissolved in maleic acid buffer:
100 mM malic acid
150 mM NaCl
Adjust pH to 7.5 (20°C) with NaOH. Store at 4°C.

Hybridizing Solution
Make fresh, store at rt. Final concentrations in H₂O.
10 mM Tris-HCl pH 7.2
70% formamide (from the deionized stock)
0.5% blocking reagent (from 10% stock)

Hybridization wash #1
10 mM Tris-HCl pH 7.2
70% formamide
0.1% BSA, must be dissolved before adding formamide.

Hybridization wash #2
0.1 M Tris-HCl pH 7.2
0.15 M NaCl
0.08% Tween-20
Add DAPI to 2nd of 3 washes:
1:3,000 DAPI from 0.5 mg/ml stock (in H₂O)

PNA probes- custom made from BioSynthesis. Store at 4°C.
TelG-Cy3: Tam-OO-TTAGGTTAGGTTAGG 3’
Tel-G stock is 53 µM in dH₂O. Use a 1:2000 dilution in H₂O.

FITC-TelC: FITC-OO-CCCTAACCTAACCTAA 3’
Tel-C is 111 µM in dH₂O. Use a 1:1000 dilution in H₂O.
*Verify concentrations with the documentation that came with your order.

PBG (in PBS)
0.2% (w/v) cold water fish gelatin (Sigma G-7765)
0.5% (w/v) BSA (Sigma A-2153)
Store in 50 ml aliquots at -20°C or filter sterilize and store at 4°C.

3000x DAPI
Dissolve 0.5 mg/ml 4’,6-diamino-2-phenylindole (Sigma D-9542) in H₂O.
Stable for at least one year at 4°C.

Embedding medium
Dissolve 20 mg p-phenylene diamine (Sigma P-6001) in 2 ml 10x PBS by vortexing. Immediately add 18 ml glycerol, mix carefully without creating air bubbles: do not vortex. Store in 1 ml aliquots at -70°C. The solution should be colorless; discard when it turns yellow or brown. *Or use ProLong Gold Antifade Reagent (Invitrogen #P36934)
Fixative
Make fresh: 3:1 MeOH and glacial acetic acid.

1. Methanol/Acetic acid fixed metaphase spreads
   1. Grow cells (e.g. HeLa) to ~40% confluence in a 10 cm culture dish.
   2. Incubate 30 min – 2 hrs in regular medium with 0.1 µg/ml demecolcin (colcemid) – the effects of the colcemid should be obvious at harvest time (rounded, refractile cells with blebby membranes).
   3. Harvest cells by trypsinization, suspend in media, and spin them down in 15 ml conical tube (5 min at 1000 rpm). Be gentle during harvesting, many of the dividing cells tend to lift off. Cells that have floated off during the harvest can be recaptured by spinning down the wash supernatant as well.
   4. Remove supernatant completely, resuspend in 5 ml of 0.075 M KCl (pre-warmed to 37°C), be gentle to prevent lysis, this step swells the cells.
   5. Incubate for up to 30 min at 37°C, invert the tubes to keep the cells suspended, check the cells during incubation and make sure they are resuspended.
   6. Spin the cells down, 5 min 1000 rpm.
   7. Decant the KCl, resuspend cells fully in the small volume of KCl that was left (by tapping).
   8. Drop by drop add 500 µl of fixative while the cells are slowly and gently mixed on a vortex (<1000 rpm).
   9. Add another 500 µl of fixative being less careful.
   10. Fill to 10 ml with the fixative and store at 4°C o/n or longer; cells can be kept at this stage indefinitely; when dealing with few cells, its better to spin them down from the first ml and then suspend them again in a small volume.
   11. When ready to drop, spin the cells down (1000rpm).
   12. Remove 9 ml of fixative and resuspend cells in the 1 ml left (may vary depending on cell number).
   13. Place a few slides in cold water. Place wet paper towels on top of a heating block set to 70°C. (We use Fisher Scientific Frosted slides #12-550-11.)
   14. Drop the resuspended cells from a couple of inches on each end of the wet slide, wash the nuclei with fresh fixative (drop fixative across the slide with a bulb and Pasteur pipette; the nuclei and chromosomes should not wash off.)
   15. Place slides for 1 min on the humidified 70-80°C heating block (place wet paper towels on the hot plate and place the slides on top of the wet paper towels).
   16. Check the slides under a regular light microscope for spreading efficiency. You should see many nuclei (all of the cytoplasmic membranes should be washed away or barely visible.) Well spread metaphase chromosomes should look like small black dots at low magnification. The arms of the chromosomes should be visible at higher magnifications. If the nuclei are too crowded or too sparse, you may need to dilute or concentrate your sample and drop the slide again.
   17. Let the slides dry o/n in a fume hood.
2. Telomeric FISH with PNA probes

(Lansdorp et.al. Hum Mol Gen, 1996,5, 685-691.)

1. Rehydrate MetOH/Acetic acid spreads in PBS (pH 7.0-7.5) 5 minutes.
2. Perform the following optional steps:
   a. Fix in 4% formaldehyde in PBS, for 2 min (dilute from 37% commercial formaldehyde).
   b. Wash in PBS 3 times, 5 minutes each.
   c. Treat with pepsin (1 mg/ml) @37°C, 10 min; pepsin prepared fresh in 10 mM glycine pH 2 and warmed up to 37°C.
   d. Wash in PBS 2 times, 2 min each.
   e. Fix in 4% formaldehyde in PBS, 2 min.
3. Wash in PBS 3 times, 5 min each.
4. Dehydrate in ethanol:
   • 5 min in 70%
   • 5 min in 95%
   • 5 min in 100%
5. Air dry slides.
6. Place 10 µl of hybridization mix onto each metaphase spread on the slide; avoid air bubbles, place the hyb mix onto the cover slip and pick the hyb mix up with the slide (cells down), then turn the slide up.
7. Denature on a 80°C hot plate, 3 min.
8. Hybridize for 2 hours, @ rt, in the dark, with wet paper towels.
9. Wash with hybridization wash #1 in small Coplin jars 2 times, 15 min each.
10. Wash with hybridization wash #2. Do this 3 times for 5 min each in small Coplin jars. Add DAPI to the second wash.
11. Dehydrate as above: 5 min in 70%, 5 min in 95%, 5 min in 100% ethanol.
13. If the probe you are using is already conjugated to a fluorescent tag, place some embedding medium onto each spread, place a cover slip on top, and seal with nail polish and store at -20°C., in the dark.

If Using Custom Probes

1. If the probe is not conjugated, for example it is biotinylated after air drying the slides, incubate 20 minutes in PBG.
2. Incubate with FITC conjugated avidin (AVIDIN DCS-FITC) (http://www.vectorlabs.com) 1:400 30 min at 37°C. (You can also amplify with the Vector ABC system).
3. Wash 3 times, 5 min in PBG, second wash with DAPI.
4. Wash with PBS, add embedding medium, seal with nail polish and store at -20°C in the dark.
5. When using a biotynylated probe, after a second alcohol dehydration step, incubate the spreads in PBG (2 times 10 minutes each) to block non-specific binding, and then with fluorescein-conjugated avidin in PBG at an adequate dilution (follow the antibody incubation steps in the IF FISH protocol).