MicroRNA Protocol for In-situ Hybridization on Frozen Sections

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I. Synthesis of DIG labeled LNA miRNA probe (miRCURY™ LNA Detection probe, ready-to-label)

Note: If using prelabeled detections probe proceed to section II.

1.1 Label the miRCURY™ LNA Detection probe:

- Label 100 pmol of the LNA oligos for miRNA detection using the DIG Oligonucleotide Tailing Kit (Roche cat # 3-353-583) miRCURY™ LNA Detection probes from Exiqon concentration: 25 µM stock = 25pmol/µL
- miRCURY™ LNA Detection probe labeling reaction:
- Add 100 pmol miRCURY™ LNA Detection probe and water to a volume of 9 µL
- Add the following on ice: 4.0 µL reaction buffer
  4.0 µL $COCl_2$-solution
  1.0 µL DIG-dUTP solution
  1.0 µL dATP solution
  1.0 µL 400 U Terminal transferase
- Mix and centerfuge briefly
- Incubate at 37°C for 30 minutes and place on ice.
- Stop reaction by adding 5µL of 0.1M EDTA (pH 8.0)

1.2 Purify probe using a Sephadex G25 Column (Amersham Biosciences # 27-5325-01)

- Resuspend column by inversion
- Snap top off, then bottom off
- Spin 1000g, 1 min in 1.5 mL tube and discard buffer
- Add probe to column
- Spin 1000g in fresh 1.5 mL tube, 4 min and retain labeled probe
- Approximate probe concentration = 30ng/ µL
- Avoid freeze thaws by aliquotting labeled probe into multiple tubes before storage at –20°C.

1.3 Check labeling efficiency and approximate concentration by dot blot:

- Dilute labeled LNA oligo and Control DIG-dUTP/dATP tailed oligo (2.5 pmol/µL - provided in Roche kit) in water
- Make the following serial dilutions:1:10, 5:50, 5:50, 5:50, 5:50
- Spot 1 µL of undiluted probe and 1 µL of each dilution on nitrocellulose.
- Air dry membrane and UV-crosslink both sides
- Equilibrate with 1xPBS for 3 min
- Block with 1% BSA in 1xPBS for 30 min at RT
- Incubate in block solution with 3 µL of anti-DIG-AP conjugated antibody for 30 min at RT (Roche Anti-Digoxigenin-AP #1093274)
- Wash 2X with 1xPBS for 10 min each
- Add 1-STEP NBT/BCIP (Pierce #34042) developer for 15-30 min
- Stop reaction with TE

II Preparing Frozen Sections for Hybridization

2.1 Tissue Preparation

- Dissect tissue and put into 4% Paraformaldehyde*/PBS O/N (overnight) at 4°C.
- 0.5M Sucrose/PBS (filter sterilized) O/N at 4°C. (place tissue directly into sucrose without washing – residual 4% Paraformaldehyde is good)
- Freeze tissue in OCT in an Ethanol/Dry Ice slurry and store blocks at –80°C until ready to use.

2.2 Cut Sections for Hybridization (Schaeren-Weimers and Gerfin-Moser, Histochemistry (1993) 100:431-440)

- Cut sections of 12-14 uM on cryostat and mount on Superfrost /Plus (Fisher #1255015) slides.
- Air dry sections for at least 20 minutes but no longer than 3 hours. (Store at –20°C)
  Some people claim best to cut tissue and hybridize on same day but slides are fine at –20°C (frost-free freezer) for months.
- Fix sections in 4% Paraformaldehyde*/PBS at 4°C for 10 minutes.
- Wash 3 times with PBS for 5 minutes each.
- Acetylate for 10 minutes in:
- 2.33 ml triethanolamine
- 500 ul acetic anhydride
- Volume up to 200 mL in water mix and use solution immediately on sections.
- Wash with 1xPBS, 3 times for 5 min each.

III. Hybridizing DIG Probes to Slides

3.1 Prepare Hybridization Mixture

Note: A final concentration of 20-40 nM is recommended.

- Prepare hybridization mixture by adding 1-2 µL of labeled probe per 200 µL of hybridization buffer. (~30ng/ µL = original conc of DIG probe).

Hybridization Buffer: D.Wilkinson In-situ Hybridization: Practical Approach, 1992

50% deionised formamide
0.3M NaCl
20 mM Tris HCL, pH 8.0
5 mM EDTA
10mM NaPO4, pH 8.0
10% Dextran Sulfate
1X Denhardt's solution
0.5 mg/mL yeast RNA
Store in aliquots at –80°C

- Heat Hybridization mixture at 65°C for 5 min to linearize probe and chill on ice.
- Add 50-100 µL of hybridization solution to slides. Spread solution over section gently with a piece of parafilm.
- Cover sections with RNase-free plastic coverslips. (Hybrislip, Schleicher & Schuell, HS40 – 40x22mm; HS22 – 22x22mm via Thomas Scientific)

• After "coverslipping", incubate slides O/N at a temperature that is 20-22°C below Tm (Tm: melting temperature) of the miRCURY™ LNA Detection probe in a chamber humidified* with:

50% formamide
1X SSC
(soaked Kimwipes)

*humidified chamber: small plastic slide box with lid (Micro Slide Box, VWR # 48444004) and soaked kimwipe placed at the bottom of box, taped closed around the edges and placed in incubator or hyb oven

3.2 Washing Slides After Hybridization (adapted from Heller et al. PNAS 95:11400-11405)

• Remove coverslips in 5X SSC at RT. (15-20 min)
• Wash slides 2 times for 30 minutes each at a temperature that is 20-22°C below Tm of the miRCURY™ LNA Detection probe in: 50% formamide
0.1% Tween-20
1X SSC
• Wash slides for 15 min in 0.2 x SSC at RT
• Wash slides for 15 min in PBS at RT

3.3 Antibody Detection (adapted from Heller et al. PNAS 95:11400-11405)

• Incubate slides for 1 hr at RT in Blocking Solution (Heat gently to get into solution):
0.5% blocking powder (Blocking Reagent, Roche # 1096176)
10% heat-inactivated goat (or sheep) serum (70°C for 30 min)
0.1% Tween-20
1X PBS
• Incubate slides for 2-3 hours at RT in blocking solution preincubated for 1 hour with AP conjugated anti-DIG Fab fragment (1:1500 – Roche #1093274).
Can use a PAP Pen (Liquid Blocker Super PAP Pen) to draw a liquid-repellent barrier around section on slide to decrease amount of Antibody solution used per slide.
Place slides in tupperware container with damp (with distilled water) paper towels on the bottom to make a humidified chamber.
• Washes: 2X 30 min in 0.1% Tween-20 in PBS
2X 20 min in PBS
• Detection:
Incubate 250 µL of 1-STEP NBT/BCIP (Pierce #34042) or BM Purple AP Substrate (Roche #11442074001) together with 2mM Levamisole on slide for 1-4 days in the dark at RT.
To perform color reaction, place slides upside-down with ~250µL of AP Substrate on top of a Styrofoam tray (can use inverted old 50mL Falcon tube rack) that is covered with two pieces of parafilm. The inverted styrofoam tray is placed into a tupperware container that has distilled water just covering the bottom (thus making a humidified container). Prevent the AP substrate from contacting air by placing a parafilm sheet over the surface of slides in order to avoid the formation of nonspecific precipitate due to oxidation of the substrate.
When desired intensity is reached, stop the reaction by transferring slides into Stop Solution (PBS pH 5.5, EDTA 1mM.)
Mount slides in a water-based medium (Glycerol Gelatin – heated approx 45°C or Aqua Mount, Lerner Labs #13800 - via Fisher #143905 - used at RT)

Troubleshooting:

- In some situations using single 3' DIG-labeled LNA oligos might decrease background compared to the use of the 3'-End DIG labeling kit (Roche cat # 3-353-575). Pre-labeled miRCURY™ LNA Detection Probes can be bought from Exiqon
- Substituting 0.1% Triton X-100 for 0.1% Tween-20 in the washes might help to cut down the background. I have found that 0.1% Tween-20 work fine for my miRCURY™ LNA Detection probes.
- If AP substrate detection results in a brown rather than purple/blue staining, it might be worth trying the following: stain O/N with 1-Step NBT/BCIP followed by O/N stain with BM AP substrate purple, wash and mount.

Buffers

*Please note: For optimal fixation it may be critical to use fresh formaldehyde solutions. Fresh 4% solutions can be made from 16%, methanol free, formaldehyde or from solid paraformaldehyde (4% w/v).

For preparation of buffers please refer to :
Molecular cloning : a laboratory manual / Sambrook, Joseph; Russell, David W. --
3rd ed. -- New York: Cold Spring Harbor Laboratory, 2001.