K. Mace, Sept 2007

RNA in situ protocol for paraffin sections

Use precautions to prevent RNase contamination of samples.
All solutions should be RNase-free (until sections have been fixed again in 4% paraformaldehyde, then just autoclaved solns are good enough).
Rinse = use glass jars
Add = pipette onto samples
All steps at RT unless otherwise noted

Materials:

Secure seal chambers – Grace Bio – item no. 10485029, or Invitrogen has them now too
Xylenes (histology grade)
EtOH (histology grade)
MeOH (histology grade)
1X PBS
1X PBT (PBS + 0.1% Tween-20) – make fresh
4% paraformaldehyde in PBS (fairly fresh, can freeze-thaw a few times)
Antibodies for detection (noted in protocol)
Proteinase K (optional) (aliquot 10-20 ul in 0.6 ml tubes, store at -20, and throw away remainder – does not survive freeze-thaw cycles)
Hybridization Solution (keep in freezer, warm amount needed to 37 degrees for use):
- 50% Formamide (molecular biology grade), e.g. Roche #1 814 320 (500 ml)
- 0.5X SSC (from autoclaved 5X stock)
- 100 ug/ml salmon testis DNA (from 10 mg/ml stock), e.g. Sigma #D-1626 (autoclaved is fine, no need to sonicate/boil)
- 50 ug/ml heparin (from 10 mg/ml stock), e.g. Sigma #H-3393
- 0.1% Tween-80 or Tween-20

Western Blocking reagent (Roche #1 921 673 (100 ml) = 5X stock
Staining buffer (MAKE FRESH, for alkaline phosphatase reaction only):
For 12.5 ml (enough for about 10 slides):
- 250 ul 5 M NaCl
- 625 ul 1M MgCl2
- 1.25 ml 1 M Tris pH 9.5
- 125 ul 10% Tween 20

water 10.25 ml

Day 1

1. Place slides in incubator at 60-70 degrees C for ~10-20 min until paraffin melts.
2. Rinse in:
   Xylenes 3x 3 min.
   50% Xylenes/50% EtOH for 1x 5 min
   100% EtOH for 2x 3 min
   100% MeOH for 2x 3 min
3. Remove slides one by one from MeOH, wipe around sections w/ Kimwipe, adhere chamber, and add 50% MeOH/50% PFA (4%) – leave on about 2 min.
4. Add 4% PFA for 1x 2 min, 1x 8 min.
5. Add PBS 2x 5 min.
6. Optional: incubate sections with 0.3% H2O2 in PBS for 30 min. (Can skip this step if HRP/Tyramide reaction will not be used).
7. Add PBT 3x 5 min.
8. Optional: Acetylation: add 0.1 M triethanolamine buffer, pH 8.0, + 0.25% acetic anhydride (MAKE IMMEDIATELY BEFORE USE!!! Only good for 5 min.). Incubate 5 min. Repeat (make fresh buffer w/ acetic anhydride). Add 2X SSC 1x 3 min., PBS 1x 3 min., PBT 1x 3 min.
9. Add Proteinase K (optional, but **do not** do this if also trying to detect protein!) (4 ug/ml) to each section for 10 min.
10. Add PBT 1x 1 min, 2x 5 min.
11. Add 50% PBT/50% hybe buffer 1x 2 min.
12. Add 100% hybe buffer 1x 2 min.
13. Bring hybe buffer to 37 degrees.
14. Incubate sections in hybe buffer in humid chamber for 1-2 hours at 55 degrees in humid chamber.
15. Dilute probe into hybe (~1-4 ul/100 ul).
16. Remove hybe buffer from sections and add hybe+probe. Incubate 16-36 hours at 55 degrees in humid chamber.

Day 2

17. Hot wash sections with warmed up hybe buffer at 55 degrees 1x 5 min, 2x 15 min.

18. Wash in 50% PBT/50% hybe buffer 1x 5 min (RT).
19. Wash in PBT 3x 5 min.
20. Wash in block (PBT + Western Blocking reagent) 1x 5 min.

** Choose EITHER colorimetric (blue ppt) reaction OR fluorescence **

For colorimetric (blue ppt) reaction:

21. Incubate in sheep anti-DIG-AP (Roche works well, determine optimal dilution 1:250-1:2000) 30 min.
22. Wash in PBT 5x 2 min.
23. Wash in fresh staining buffer 2x 2 min.
24. Incubate in stain (Per 1 ml = staining buffer + 3.72 ul 20 mg/ml BCIP + 5 ul 75 mg/ml NBT, determine optimal concentration). Signal will start to appear in 5-30 minutes.
25. When signal looks good, remove chambers and rinse in PBT 3x 2 min., PBS 2x 1 min.
26. Optional: Counterstain in nuclear fast red.
27. Wash in water, 3x 5 min., or until stain is nice.
28. Remove chambers and dehydrate by incubating in slide trays:
    50% EtOH/50% H2O 1x 3 min
    75% EtOH/25% H2O 1x 3 min
    100% EtOH 3x 3 min
    Xylenes 2x 3 min
29. Mount in Permount.

For Fluorescence:

21. Add sheep anti-DIG IgG (Roche #1 333 089, 1:400), incubate 30 min.
22. Wash in PBT 5x 2 min.
23. Add anti-sheep-Alexa-555 (#A 21436, Molecular Probes/Invitrogen, 1:400), incubate 30 min.
24. Add PBT 2x 5 min.
25. Counterstain with DAPI (1:1000 of 5 mg/ml stock), 5 min.
26. Remove chambers and rinse in PBT 2x 5 min., PBS 1x 5 min.
27. Mount in Fluorguard (BioRad), or Vectashield (if it has DAPI in it then skip above DAPI staining).