In Situ Hybridization Protocol on Slides.

Dewax slides :

1. Toluene 5'
2. Toluene 10'
3. 100% EtOH 5'
4. 80% EtOH 5'
5. 70% EtOH 5'
6. DEPC-PBST 5'
7. Write circle with Super PAP Pen (Zymed)
8. Proteinase K (7.5 μg/ml) in DEPC-PBST for 5'
9. DEPC-PBST 5'
10. Post-fix in 4% paraformaldehyde + 0.2% glutaraldehyde for 20'
11. DEPC-PBST 5'

Hybridization of Probe

- Add pre-warmed (to 68°C) hybridization buffer to slides.

Hybridization Buffer	**50 ml**	**10 ml**	**Final Concentration**
Deionized Formamide	25 ml	5 ml	50 %
20X SSC Buffer	12.5 ml	2.5 ml	5 x
10mg/ml tRNA	250 μl	50 μl	0.05mg/ml
10% SDS	5 ml	1 ml	1% SDS
100mg/ml Heparin	25 μl	5 μl	0.05 mg/ml
DEPC H_2O	7.225 ml	1.445 ml	

- Replace once with fresh hybridization buffer and prehybridize at 70°C for 1 hour in moist chamber. Moist chambers : put a piece of Whatmann paper and broken 5 ml pipets into the box, to form a platform for the slides. Pour enough moist chamber solution into the box to cover the bottom but don't fill it over the pipette base. Place a beaker of dH_2O in the hybridization oven with the moist chamber to keep the humidity level stable. This will prevent the moist chamber solution from evaportating over night.
- Mix 2μL of the probe into 500μL of hybridization buffer.
- Put 100 μl of probe mix on slide, cover with coverslip (ac) and hybridize overnight at 70°C.
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Moist chamber solution.

	Final concentration	For 50 ml
deionized formamide	50 %	25 ml
20 x SSC	2 x	5 ml
dH_2O		20 ml

WASHES

1. Wash in 50 % deionized Formamide/2 x SSC/ 0.1 Tween 20 for 15' at 65°C. Coverslips should fall off during this wash. If not, gently help them to fall off. Do not pull them off --- this will destroy the tissue.
2. Wash in the same solution 2 x 30' each at 65°C.
3. Wash 3x 5' with TBST/2mM Levamisole (0.5mg/mL).

BLOCKING AND HYBRIDIZATION OF DIG ANTIBODY

- Make Embryo Blocking Solution and Antibody Blocking Solution
  - Blocking Solution — Final Concentration
    - 800μL 10% BM Blocking Reagent — 2%
    - 400μL Heat Inactivated Fetal Bovine Serum — 10%
    - 2.8mL TBST/2mMLevamisole
  - Antibody Solution — Final Concentration
    - 400μL 10% BM Blocking Reagent — 2%
    - 40μL Heat Inactivated Fetal Bovine Serum — 1%
    - 3.16mL TBST/2mMLevamisole
- Dilute the anti-DIG antibody at 1:2000 in the antibody solution.
- Incubate the slides for 30' in blocking solution.

COLOR REACTION

- Wash the slides 3x 5' with TBST/2mM levamisole.
- Wash 2x 5' in NTMT/2mM levamisole.
  - NTMT/Alkaline Phosphatase Buffer — Final Concentration
    - 2ml 5M NaCl — 100mM
    - 10mL 1M Tris pH 9.5 — 100mM
    - 5mL 1M MgCl — 50mM
    - 100μL Tween-20 — 0.1%
    - 82.9ml Autoclaved H_2O
- Bring BM Purple solution to 37°C.
- Incubate the slides in BM purple (4h-24h at RT) or shorter at 37°C.
- Wash in PBS.

- **10% BM Blocking Reagent**
  Boehringer-Mannaheim Catalog # 1098176
  - Malic Acid Buffer Final Concentration
    - 8g Maleic Acid (disodium salt Sigma#M9009) 100mM
    - 8.76g NaCl 150mM
    - pH to 7.5 with NaOH.
    - Final Volume- 500mL
  - Add 50g of Blocking reagent to Maleic Acid Buffer to bring to a final concentration of 10% w/v. Aliquot and autoclave.
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- **10X TBS for 100mL**
  - 8g NaCl
  - 0.2g KCl
  - 3g Tris pH 7.6
  - TBST is 1X TBS with 0.1% Tween-20
  - TBST/2mM Levamisole has 0.5mg/ml Levamisole

- **10X PBS for 100mL**
  - 8g NaCl
  - 0.2g KCl
  - 1.44g Na_2HPO_4
  - 0.2g KH_2PO_4

We have also a protocol to enhance the staining reaction

The method for enhancing the colour reaction is as follows:

After the final three NTMT washes (normally done in preparation for the BCIP/NBT reaction), make a solution of NTMT containing 10% PVA (polyvinyl alcohol; Sigma P-8136) as follows:

For every 10ml of this solution, add
- 9.16 ml distilled water,
- 0.5 ml Tris-pH9.5
- 340 microlitre of 3M NaCL

Heat or microwave this solution to 90 C (doesn't matter if boils slightly), then cool to about 60 C and add 1g of PVA powder. Shake vigorously and leaveto mix with a stirring rod in the solution on a hot plate until it goes into
solution. Should only take about 10-15 mins.

-Cool the solution to RT.

-then add 12.5 microlitre of 2M MgCl2 (therefore final MgCl2 concentration is 5mM) and mix well.

The solution will be viscous with lots of bubbles on top. Just allow the bubbles to settle down a little!

Then for EVERY 10 ml of this solution add: 2.5 microlitre of NBT (from a 75mg/ml stock) and 1.7 microlitre of BCIP (from a 50 mg/ml stock).

The NBT and BCIP used here seems very low compared to standard protocols but
it works, so don't add any more than recommended!

Allow the reaction to develop at room temperature or up to 28 C. I find that this reaction cuts the normal developing times by about 3/4! so that a probe that normally takes 4 hours to come up will be coming up in 1 hour! So examine the reaction regularly.

This method has worked both for whole mount and on sections. Obviously larger volumes would have to be made up for sections.

Good luck

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