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Combined Digoxigenin-labeled in situ hybridization/ Immunohistochemistry protocol (for fixed frozen cryostat sections)

A. Digoxigenin-UTP labeling of cRNA antisense probe

Refer to laboratory protocol and Boehringer-Mannheim product specifications.

B. Cryostat sectioning & Post-fixation

1. 4% paraformaldehyde immersion fixation @ 4°C

2. 10%, 15%, 20% sucrose/1XPBS gradients @ 4°C

3. Embed in OCT and freeze over dish containing 2-methylbutane over dry ice

4. Store @ -80°C

C. Pretreatment

1. Previously fixed slides are placed in 4% paraformaldehyde/PBS for 5 min.

2. Wash in 1XPBS/DEPCddH_20 3x 5 min.

3. Wash in autoclaved DEPCddH_20 1x 5 min.

4. Place in freshly prepared acetylation solution for 10 minutes as follows (DEPCddH_20 200 ml, triethanolamine [TEA, Fisher] 3 ml, 0.5 ml acetic anhydride [AcAn, Fisher]). Place slides in stirring TEA and add AcAn, stir x5 min, still x5 min.

nb. it is important to adjust the pH of the TEA to 8.0 with HCl prior to the addition of the acetic anhydride.

5. see 3.

6 Place in 3X SSC/ 50% formamide (Fisher) and subject to hybridization without drying. Can store sections in this solution at RT for a few hours prior to hybridization.

D. Hybridization

1. Hybridization solution:

50% formamide (Ultrapure Gibco BRL)
1X Denhardt's solution
3X SSC
10mM EDTA
10% Dextran sulfate

1. Hybridization solution: (continued)

500 μg/ml yeast tRNA
500 μg/ml salmon sperm DNA (heat denatured, BRL)

2. Add 1 μl of a 50 μl reaction Dig-UTP label cRNA probe per 100 μl hybridization solution.

3. Heat probe solution @ 80°C for 5 min, then place on wet ice for 2 min.

4. Place 80-90 μl of the solution on a precut parafilm coverslip.

5. Pick up coverslips with inverted glass slides, avoid air bubbles.

6. Set slides in hybridization chamber humidified with 3X SSC, 50% formamide.

7. Incubate @ 55°C for 16- 20 hours.

E. Washing

1. 5X SSC @ 55°C for 5-10 min to remove coverslips (prewarm wash solutions overnight in hybridization oven).

2. 2X SSC/50% formamide @ 55°C for 45 min.

3. NTE (10 mM Tris Hcl pH8.0, 1 mM EDTA, 500 mM NaCl) @ 37°C x 15 min.

4. NTE + 20 μg/mL RNAse A @ 37°C x 30 min.

5. NTE @ 37°C x 15 min.

6. 0.5X SSC/50% formamide @ 55°C x 45 min.

7. 0.5X SSC without formamide @ RT x 5 min.

F. Probe Detection

1. Buffer 1 [TBS(0.1 M Tris HCl pH 7.6, 150 mM NaCl)] 3x 5 min @ RT

2. Block in TBS/10% FBS, heat inactivated x 60 min @ RT

3. Incubate O/N @ 4°C in 1:2000 dilution of anti-digoxigenin-Fab-AP conjugate (BMB 1093274) in TBS/10%FBS (fetal bovine serum, heat inactivated). No coverslip is necessary. Use 250 μl/slide.

4. Wash in Buffer 1 3x 10 min @ RT.

5. Buffer 3 (100 mM Tris HCl pH**9.5**, 50 mM Mg Cl_2, 100 mM NaCl) 1x 10 min. Add Levamisole 0.5 mg/mL to block endogenous alkaline phosphatase

6. Buffer 3, NBT 45 μL/10 mL (BRL), BCIP 35 μL/10 mL (BRL), + Levamisole 0.5 mg/mL (Sigma). Develop in **dark** @ RT. May leave O/N @ 4°C.

7. Stop reaction in TE pH 8.0 (10 mM Tris HCl pH8.0, 1mM EDTA).

8. Post-fix in 4% PFA/PBS x 20 min @ RT.

9. Wash in 1X PBS 3x 5 min.

G. Immunohistochemistry

1. Blocking solution:

   5% normal goat serum
   1X PBS (final concentration)
   0.2% Triton X-100
   0.1% Bovine serum albumin (use Fraction V lyophilized powder)
   0.02% sodium azide (nb. highly toxic)

2. Block for either 4 hours @ RT or overnight @4°C

3. Primary antibody: Dilute in blocking solution and incubate overnight @4°C.
   [α–DLX-1/FL 1:15]
   [α–DLX-2/C199* 1:250]

4. Wash in 1XPBS 3x 5 min @ RT; omit Triton in wash solutions since Dig-AP may "leak" out.

5. Secondary antibody: Dilute biotinylated goat anti-rabbit IgG (Vector) in blocking solution 1:200. Incubate 1-2 hours @ RT.

6. See 4.

7. Prepare 250 mL of 0.3% hydrogen peroxide (Fisher) in 1XPBS. Quench endogenous peroxidases by washing for 15-30 min. @ RT. (Peroxide treatment following incubation with secondary antibody provides improved reduction of background staining).

8. See 4.

9. Prepare tertiary substrate (Vector 'Elite' ABC):
add 2 drops 'A' to 5 mL 1XPBS, mix well, then add 2 drops 'B' and mix. Let stand at least 30 min. @ RT prior to use.

10. Add 200 μL ABC solution to slides. Incubate 30-60 min. @ RT.

11. See 4.

12. Prepare DAB chromogenic substrate (Vector). nb. Add peroxide solution just prior to use. Waste and excess DAB treated with 2% bleach prior to discarding in special waste container.

13. Add 200 μL DAB. Allow staining for 1-30 min., usually 2-10 min.
Observe under microscope.

14. Stop reaction in water for 5 min. @ RT.

15. Dehydrate in graded ethanols: 50, 75, 85, 95, 100, 100% x 2 min. each.

16. Xylene x2 for at least 2 min. each.

17. Mount with coverslip using Permount (Fisher).

18. Alternative mounting protocol (omit steps 15 & 16): Directly coverslip using Aqua-Polymount (Polysciences) and seal with nail polish enamel.

H. Results

This protocol works best when studying nuclear transcription factors, especially those which are highly expressed in a region-specific manner.

The **Dig-AP** conjugate provides a blue-purple color reaction in the **cytoplasm**; the **DAB** substrate gives a brown **nuclear** stain.