

Immunolocalization by the peroxidase anti-peroxidase (PAP) method after *in situ* hybridization^a

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Reagents

- ◆ 3% hydrogen peroxide: made fresh from a 30% stock solution stored at 4 °C
- ◆ DAB staining solution: 0.5 mg/ml diaminobenzidine in PBT.

DAB is a potent carcinogen. All contaminated disposable materials should be incinerated and glassware soaked in 6% sodium hypochlorite solution

- ◆ PAP complex: peroxidase-conjugated rabbit anti-peroxidase (PAP, Jackson Immuno-Research Laboratories)
- ◆ PFA fix: paraformaldehyde is dissolved in PBS at 65°C. If it does not readily dissolve add a drop or two of 1 M NaOH solution to pH 7.5. It should be cooled to 4°C and used within 2 days
- ◆ Blocking solution for antibodies: 1x PBS, 0.1% Tween-20, 2 mg/ml BSA (BDH), 5% sheep serum (Gibco BRL), 1% dimethylsulphoxide DMSO (Merck-BDH)
- ◆ Primary antibody raised in rabbit against the tissue antigen
- ◆ Secondary antibody: goat anti-rabbit IgG 'bridging antibody' (Jackson ImmunoResearch Laboratories)

Method

- 1 Perform the *in situ* hybridization as described in [Synthesis of digoxigenin or fluorescein labelled RNA probe](#), [Fixation and pre-treatment of embryos for whole mount hybridization](#), and [Whole mount hybridization, washing, and detection of probe \(method 1\)](#). Use an antisense RNA probe labelled with digoxigenin and an antibody conjugated with alkaline phosphatase.
- 2 Stain with NBT/BCIP as described in [Zebrafish or Drosophila two colour whole mount *in situ* hybridization - staining with DAB and BCIP/NBT](#); [Two colour *in situ* hybridization - sequential alkaline phosphatase staining with chromogenic substrates of zebrafish embryos](#), and [Two colour *in situ* hybridization - sequential alkaline phosphatase staining with chromogenic substrates of chick, mouse, and *Xenopus* embryos](#).
- 3 Stop the staining by rinsing in PBT and refix for 20 min in PFA fix in PBS.
- 4 Incubate in blocking solution for 30 min at room temperature.

- 5 Incubate 5 h at room temperature in a 1:2000 dilution of primary rabbit antibody in blocking solution.
- 6 Wash for 2 h with PBT (eight times for 15 min each).
- 7 Incubate overnight at 4 °C in blocking solution containing a 1:100 dilution of secondary antibody in blocking solution.^b
- 8 Wash for 2 h with PBT (eight times for 15 min each).
- 9 Incubate for 5 h in blocking solution containing a 1:400 dilution of PAP complex.
- 10 Wash for 2 h with PBT (eight times for 15 min each).
- 11 Incubate for 2 min in DAB staining solution.^c
- 12 Add 1/1000 volume of 3% hydrogen peroxide.
- 13 Monitor staining and stop by rinsing thoroughly with PBT.

Notes

- a This is modified from a method described in Jowett, T. (1996). *Tissue in situ hybridization: methods in animal development*. Publ. Wiley and Sons, NY.
- b It is important in the PAP technique for the bridging antibody to be applied in excess. This way one arm of the divalent Fab portion of the immunoglobulin molecule can bind the primary antibody, while the other arm is free to bind the PAP complex.
- c TrueBlue™ can be used as an alternative substrate to DAB. The TrueBlue™ horse-radish peroxidase substrate is more sensitive than DAB and so the titre of the antibody can be decreased by 10- to 50-fold. However, the blue precipitate formed is less stable, being partially soluble in alcohol and water. If used in a two colour *in situ* hybridization with horse-radish peroxidase- and alkaline phosphatase-conjugated antibodies, the phosphatase should be stained first and then the peroxidase. Otherwise the blue precipitate will be lost or weakened.