

Immunolocalization with a horse-radish peroxidase-conjugated secondary antibody after *in situ* hybridization

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Reagents

- ◆ 3% hydrogen peroxide: made fresh from a 30% stock solution stored at 4 °C
- ◆ Anti-mouse IgG-HRP: goat anti-mouse IgG conjugated with horse-radish peroxidase
- ◆ 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)
- ◆ 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
- ◆ 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
- ◆ Primary IgG monoclonal antibody

Method

- 1 Perform the *in situ* hybridization as described in [Synthesis of DIG or fluorescein labelled RNA probes](#), [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.
- 4 Incubate in blocking solution for 30 min at room temperature.
- 5 Incubate for 5 h at room temperature in a 1:2000 dilution of primary monoclonal antibody.^a
- 6 Wash for 2 h with PBT (eight times for 15 min each).

- 7 Incubate overnight at 4 °C in blocking solution containing a 1:5000 dilution of anti-mouse IgG-HRP.
- 8 Wash for 2 h with PBT (eight times for 15 min each).
- 9 Incubate for 2 min in DAB staining solution.^b
- 10 Add 1/1000 volume of 3% hydrogen peroxide.
- 11 Monitor the staining reaction and stop by rinsing thoroughly with PBT.
- 12 Fix the stain by incubating in PFA fix for 20 min.

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 The dilution of primary antibody used is dependent on the particular antibody and may have to be optimized for the highest signal-to-noise ratio.
- c TrueBlue™ can be used as an alternative substrate to DAB. The TrueBlue™ horse-radish peroxidase substrate is more sensitive than DAB, so decrease the titre of the secondary antibody 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 before the peroxidase, otherwise the blue precipitate will be lost or weakened.