

Immunolocalization by avidin biotinylated enzyme complex staining of zebrafish embryos after *in situ* hybridization^a

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Reagents

- ◆ 3% hydrogen peroxide: made fresh from a 30% stock solution stored at 4 °C
- ◆ Benzylbenzoate:benzyl alcohol mixed in the ratio 2:1
- ◆ DAB stock solution: 5 mg/ml diaminobenzidine in 10 mM Tris-HCl pH 7
- ◆ Primary antibody raised in rabbit against the tissue antigen
- ◆ Blocking solution: 5% goat serum (supplied with the Vector Elite™ rabbit IgG kit), 1% DMSO, 0.1% Tween 20 in PBS
- ◆ Vector Elite™ rabbit IgG kit, contains reagents A and B for forming the avidin-biotinylated peroxidase complex, biotinylated goat anti-rabbit, and goat serum (tested biotin-free)

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 rabbit antibody in blocking solution.
- 6 Wash four times for 20 min each with blocking solution.

- 7 Incubate in a 1:2000 dilution of secondary antibody (biotinylated goat anti-rabbit, Vector Elite™ rabbit IgG kit) for 4–5 h at room temperature or 4 °C overnight.
- 8 Wash four times for 20 min each with blocking solution.
- 9 Rinse briefly with PBT.
- 10 Make the AB complex. Add 40 µl of reagent A (avidin DH) to 5 ml of blocking solution. Mix and add 40 µl of reagent B (biotinylated enzyme). Mix and incubate for 30 min at room temperature.
- 11 Incubate the embryos in the AB complex for 45–60 min.
- 12 Wash four times for 25 min each with blocking solution.
- 13 Wash briefly in PBT.
- 14 Incubate for 2 min in 2 ml PBT plus 100 µl of DAB stock solution.
- 15 Add 4 µl of 3% H₂O₂. Allow to stain until the signal appears (usually a few minutes). Stop the reaction by washing the specimen in PBS.
- 16 Dehydrate by putting the specimen in 100% methanol. Change once (twice for 10 min each is enough). Clear the embryos by transferring to a 2:1 mixture of benzylbenzoate:benzyl alcohol.

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.](#)