

Chick, mouse, and *Xenopus* two colour whole mount *in situ* hybridization staining with Fast Red and TrueBlue^a

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Equipment and reagents

- ◆ Orbital shaker or roller
- ◆ Anti-DIG–HRP: sheep anti-digoxigenin Fab fragments conjugated with horse-radish peroxidase, make a working dilution in blocking solution
- ◆ Anti-FLU–AP: sheep anti-fluorescein Fab fragments conjugated with alkaline phosphatase, make a working dilution in blocking solution
- ◆ Blocking solution: MABTB with 20% heat treated sheep serum (56 °C for 30 min)
- ◆ MABT buffer: 100 mM maleic acid (Sigma), 150 mM NaCl, 0.1% Tween-20, pH 7.5
- ◆ MABTB: MABT + 2% Blocking powder. Make a 10% stock solution by heating 1 g of Blocking powder (Boehringer) in 10 ml of MABT. Dissolve, autoclave and store in aliquots at -20°C
- ◆ Pre-stain buffer: 100 mM Tris–HCl pH 8.2
- ◆ TrueBlue™ staining solution (KPL, Kirkegaard & Perry Laboratories)
- ◆ Vector Red™ staining solution (Vector Labs), mix stock solutions as described with kit

Method

- 1 Carry out probe synthesis, tissue fixation and pre-treatment, hybridization, and washing 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 \(method 1\)](#). After steps 1–4 in [Whole mount hybridization, washing, and detection of probe \(method 2\)](#), replace the MABT with blocking solution (MABTB).^b
- 2 Incubate for 1 h at room temperature.
- 3 Replace the MABTB with blocking solution and incubate for a further 1–2 h.
- 4 Incubate for at least 2 h in a 1:2000 to 1:8000 dilution (0.375–0.094 U/ml) of pre-absorbed anti-FLU–AP in blocking solution.
- 5 Rinse three times with MABT.
- 6 Wash three times for 60 min each with 10–20 ml MABT, by rolling at room temperature.
- 7 Wash twice for 10 min each with 100 mM Tris–HCl pH 8.2.

- 8 Incubate in Vector Red™ staining solution for a few hours to overnight.
- 9 When the colour has developed to the desired extent wash three times with PBT.
- 10 Replace PBT with blocking solution and incubate for a further 1 h.
- 11 Incubate for at least 2 h with pre-absorbed anti-DIG–HRP at a dilution of 1:2000 (0.075 U/ml) in blocking solution.
- 12 Rinse three times with MABT.
- 13 Wash three times for 60 min each with 10–20 ml MABT, by rolling at room temperature.
- 14 Replace the MABT with TrueBlue™ staining solution.^c
- 15 Monitor staining and photograph. The TrueBlue™ colour may fade once the staining solution is removed. It can be recovered by placing back in staining solution.

Notes

- a Modified from Jowett, T. and Lettice, L. (1994) *Trends Genet.* **10**: 73-74 and Jowett, T. (1996). *Tissue in situ hybridization: methods in animal development*. Publ. Wiley and Sons, NY. Harland R.M. (1991) *Methods in Cell Biology* **36**: 685-695. Lamb, T.M., Knecht, A.K., Smith, W.C., Stachel, S.E., Economides, A.N., Stahl, N., Yancopolous, G.D., and Harland, R.M. (1993) *Science* **262**: 713-718. Henrique D., Adam, J., Myat, A., Chitnis, A., Lewis, J. and Ish-Horowicz, D. (1995) *Nature* **375**: 787-790.
- b Block sample completely: intense endogenous peroxidase activity may be blocked by incubating slides or tissues for 30 min in 0.3% (w/v) H₂O₂ in 100% methanol, followed by a 10–15 min rinse in 0.1 M Tris–HCl pH 7.6.
- c TrueBlue™ is 10–50 times more sensitive than DAB. Hence initially use a tenfold dilution of the peroxidase-conjugated antibody. If a high background is seen repeat with a reduced antibody titre. Also, the stain may fade if the embryos are removed from the staining solution or fixed. If this occurs re-equilibrate in PBT and restain.