

## Two colour *in situ* hybridization - sequential alkaline phosphatase staining with chromogenic substrates of chick, mouse, and *Xenopus* embryos<sup>a</sup>

T. Jowett

Department of Biochemistry and Genetics, The Medical School, The University, Newcastle upon Tyne NE2 4HH, UK.

### Equipment and reagents

- ◆ Orbital shaker or roller
- ◆ AP inactivation solution: 0.1 M glycine-HCl pH 2.2, 0.1% Tween-20.
- ◆ Blocking solution: MABTB with 20% heat treated sheep serum (56 °C for 30 min)
- ◆ Anti-FLU-AP: sheep anti-fluorescein Fab fragments conjugated with alkaline phosphatase, make a working dilution in blocking solution
- ◆ Fast Red tablets (alkaline phosphatase substrate; Boehringer): each tablet contains 0.5 mg naphthol substrate, 2 mg Fast Red chromogen, and 0.4 mg levamisole. Store tablets at -20°C. Dissolve one tablet in 2 ml of 100 mM Tris-HCl pH 8.2. Use the solution within 30 min

**Wear gloves and use plastic forceps to handle the tablets.**

- ◆ 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
- ◆ Anti-DIG-AP: sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase, make a working dilution in blocking solution
- ◆ NBT/BCIP staining solution. **NBT** (4-nitro blue tetrazolium chloride; Boehringer ): dissolve at 75 mg/ml in 70% dimethylformamide. **BCIP** 5-bromo-4-chloro-3-indolyl-phosphate also known as X-phosphate 4-toluidine salt; (Boehringer): dissolve at 50 mg/ml in dimethylformamide. Store both solutions in aliquots at -20°C. To make the staining solution add 4.5 µl of 75 mg/ml NBT in 70% dimethylformamide and 3.5 µl of 50 mg/ml BCIP in dimethylformamide to 1 ml of NTMT buffer.
- ◆ NTMT buffer: 100mM NaCl, 100mM Tris-HCl pH 9.5, 50mM MgCl<sub>2</sub>, 0.1% Tween-20 (if necessary, add levamisole to 5mM). Make from concentrated stock solutions on the day of use (the pH will decrease on storage, due to absorption of carbon dioxide)
- ◆ 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
- ◆ Pre-stain buffer: 100 mM Tris-HCl pH 8.2, 0.1% Tween 20
- ◆ Sigma *Fast*<sup>TM</sup> Fast Red (Sigma) dissolve a buffer tablet in water, add a stain tablet, and use immediately

- ◆ Vector™ Red staining solution (Vector Labs); mix stock solutions as described with kit

## Method

- 1 Carry out probe synthesis, tissue fixation and pre-treatment, hybridisation, 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 \(method 2\)](#). After steps 1–4 in [Whole mount hybridization, washing, and detection of probe \(method 2\)](#), replace the MABT with blocking solution (MABTB).
- 2 Incubate for 1 h at room temperature with gentle shaking or rolling.
- 3 Replace with blocking solution and incubate for a further 1–2 h with gentle shaking or rolling.
- 4 Replace the blocking solution with a 1:5000 dilution of pre-absorbed anti-FLU-AP and incubate overnight at 4 °C.
- 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 at least twice for 10 min each in 100 mM Tris-HCl pH 8.2.
- 8 Incubate with Fast Red, Sigma *Fast™* Fast Red, or Vector™ Red staining solution. Rock for the first 20 min and then transfer to 24-well plates for observation.
- 9 When the colour has developed to the desired extent (1 h to overnight) wash three times with PBT.
- 10 Inactivate the alkaline phosphatase by incubating for 30 min in AP inactivation solution. Then incubate in PFA fix overnight at 4 °C to preserve the stain.
- 11 Repeat the block by incubating in blocking solution for 1–2 h.
- 12 Replace with fresh solution containing a 1:2000 to 1:5000 dilution of pre-absorbed anti-DIG-AP and incubate overnight at 4 °C or 4 h at room temperature.
- 13 Rinse briefly three times with MABT.
- 14 Wash three times for 60 min each with 10–20 ml MABT, by rolling at room temperature.
- 15 Wash at least twice for 10 min each in NTMT.
- 16 Incubate with 1.5 ml of NBT/BCIP staining solution.<sup>b</sup>
- 17 Rock for the first 20 min and then transfer to 24-well plates for observation. Development is faster at 37 °C.

## Notes

- a This protocol is modified from 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. Knecht, A.K., Good, P.J., Dawid, I.B., and Harland, R.M. (1995) *Development* **121**: 1927-1936.
- b Increasing the concentration of Tween 20 from 0.1% to 1% gives a darker blue/purple precipitate.