

## Two colour *in situ* hybridization - sequential alkaline phosphatase staining with chromogenic substrates of zebrafish embryos<sup>a</sup>

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### Reagents

- ◆ Anti-DIG-AP: sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase, 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
- ◆ AP inactivation solution: 0.1 M glycine-HCl pH 2.2, 0.1% Tween-20
- ◆ 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)
- ◆ 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.**

- ◆ 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>™</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
- ◆ Washing solution: 2 mg/ml BSA, 1% DMSO in PBT

## Method

- 1 After hybridization and washing off unbound probes as described in steps 1–5, [Whole mount hybridization, washing, and detection of probe \(method 1\)](#), replace the PBT with blocking solution. Incubate at room temperature for 1 h.
- 2 Replace the blocking solution with a 1:5000 dilution (0.15 U/ml) of anti-FLU-AP in blocking solution. Incubate for 2 h.
- 3 Wash for 2 h in washing solution (eight times for 15 min each).
- 4 Equilibrate three times for 5 min each in pre-stain buffer (100 mM Tris-HCl pH 8.2, 0.1% Tween 20).
- 5 Stain with Vector™ Red, Fast Red, or Sigma *Fast™* Fast Red.
- 6 Stop the reaction by washing in PBT.
- 7 Rinse in PBT, and heat to 65 °C for 30 min to inactivate the alkaline phosphatase if stained with Vector™ Red. For Fast Red (Boehringer) or Sigma *Fast™* Fast Red incubate in AP inactivation solution for 30 min, and then thoroughly wash in PBT.
- 8 Fix the stain in PFA fix for 20 min.
- 9 Block with blocking solution for 60 min.
- 10 Incubate for 2 h with a 1/5000 dilution (0.15 U/ml) of anti-DIG-AP in blocking solution.
- 11 Wash eight times for 15 min each in PBT to remove unbound antibody.
- 12 Equilibrate for three times for 5 min each in NTMT buffer.
- 13 Visualize the signal by incubating in NBT/BCIP staining solution.
- 14 Stop the reaction by rinsing in PBT and fixing with PFA fix for 20 min.

## Notes

- a This protocol is modified from Jowett, T. and Lettice, L. (1994) *Trends Genet.* **10**: 73-74 and Jowett, T. (1996). *Tissue in situ hybridisation: methods in animal development*. Publ. Wiley and Sons, NY. Jowett, T, and Yan, Y-L. (1996) *Trends Genet. Tech. Tips Online*. Jowett, T., Mancera, M., Amores, A., and Yan, Y-L. (1966) In *In situ hybridization: Laboratory Companion*. p. 91. (ed. M. Clark) Chapman and Hall. Jowett, T. and Yan, Y-L. (1966) In *A Laboratory Guide to RNA: Isolation, Analysis, and Synthesis*. p.381. (ed. P. A. Krieg) Wiley-Liss, NY.