

Wholemount *in situ* hybridisation with LNA probes

Refs: Sweetman et al, Dev Dyn, 235(9), 2185-91 (2006), Sweetman et al, Dev. Biol. Jun 21(2008)

Notes

Use double DIG labelled oligos (labelled at both 5' and 3' ends) from Exiqon. Don't bother buying unlabelled LNA and doing your own labelling as it will be much less efficient. Exiqon will custom synthesise double labelled oligos on request. 250pmol (1 tube) of LNA should be added to 12.5mL hyb mix and stored at -20°C.

The hyb temp should be determined empirically. Start with the calculated melting temp -22°C.

We have experienced that pre-absorbtion of the probe against whole embryos at the hyb temp improves the specificity of the hybridisation. In addition we experience that the probes get better with repeated use, we therefore recommend a preabsorbtion step and to keep the used hyb mix.

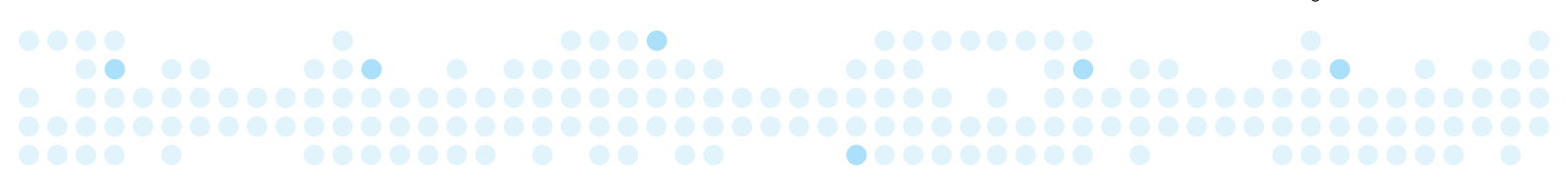
When developing colour often no staining is seen at first; when the staining develops it is hidden in diffuse soluble staining, which will be removed by washes in 5 X TBST. After the wash step the colour reaction can be resumed. Several cycles of colour reaction and washing may be required (depending on the probe) to obtain an optimal signal to noise ratio. Generally it is a good idea to wash overnight in 5 X TBST between colour reactions as this will prevent background.

Method

- Harvest embryos and fix at 4°C (min overnight) in 4% PFA (paraformaldehyde) in PBS
- Wash twice in PBST (PBS + 0.1% Tween 20)
- Wash in 50% MeOH / PBST
- Wash twice in 100% MeOH
- Store at -20°C (min. o/n)
- All washes at least 5 mins, longer for larger embryos

- Rehydrate through 75%, 50%, 25% MeOH / PBST
- Wash twice in PBST

- Proteinase K (25-30 min)- for younger embryos (incl. *Xenopus*) this step can be skipped
- 15 µg/mL prot K (e.g. for chicken embryos HH25-28)
- 20 µg/mL prot K (e.g. for chicken embryos > HH28)
- Rinse twice in PBST, fix in 4% PFA / 0.1% Glutaraldehyde, 20 mins at RT
- Rinse and wash for 5 mins in PBST



Hybridisation

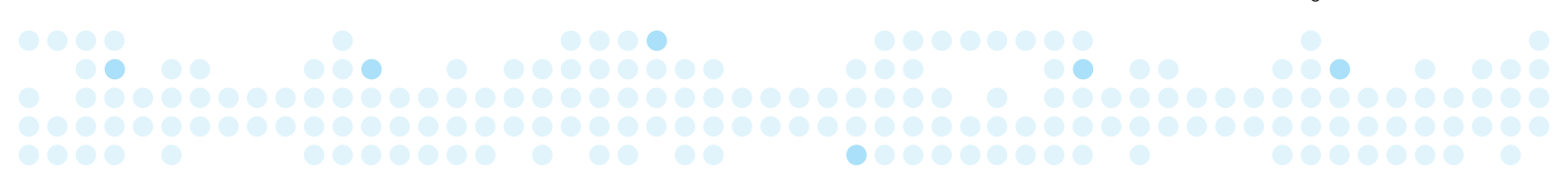
- Hyb mix: 50% formamide, 1.3 X SSC pH5 with citric acid, 5 mM EDTA, 50 µg/mL yeast RNA, 0.2% Tween-20, 0.5% CHAPS, 100 µg/mL Heparin
- Wash embryos for 10 mins. in 50% PBST / hyb mix
- Wash 10 mins with hyb mix
- Replace hyb mix and prehybridise for at least 2 hours at hyb temp
- Add prewarmed LNA in hyb mix and hyb overnight at hyb temp
- Wash 10 mins in hyb mix at hyb temp
- Wash 2 x 1 hr in wash sol. (50% formamide, 1 X SSC, 0.1% Tween-20) at the hyb temp. (Larger embryos may need longer washes and can be washed o/n without any problems)
- Wash in 50% wash sol / MABT (100mM maleic acid, 150mM NaCl, 0.1% Tween-20, pH 7.5) at hyb temp for 10 mins
- Wash 2 x 30 mins in MABT
- Block 1hr in 2% Roche blocking reagent in MABT
- At least 1 hr 2% blocking reagent + 20% goat serum in MABT
- Incubate with anti-Dig AP (dil 1:2000) o/n in 2% blocking reagent / 20% goat serum
- Rinse x 3 in MABT
- Wash 6 x 1hr (approx – this is flexible) in MABT
- Wash o/n in MABT

Color reaction

- Wash 2 x 10 mins in NTMT (100mM NaCl, 50mM MgCl₂, 100mM Tris pH9.5, 1% Tween 20)
- Incubate in NBT / BCIP (9µL NBT at 75 mg/mL in 70% DMF / 7µL of BCIP at 50 mg/mL in 100% DMF per mL of NTMT)
- Monitor reaction – when background appears wash in 5 X TBST (40g NaCl, 1g KCl, 125mL 1M Tris pH 7.5, 50mL Tween-20 in 1L) until diffuse soluble staining is gone
- Repeat colour reaction – keep alternating staining and TBST washes until pattern is developed
- Fix in 4% PFA – add 0.025% sodium azide for long term storage at 4°C

For double *in situ*

- Use solutions as above. First hybridise your normal antisense RNA probe (FITC labelled if using DIG LNA) at 65°C, then wash (min 2 x 1hr) in wash buffer (50% formamide, 1XSSC, 0.1% Tween-20) at 65°C. Then re-equilibrate in hyb buffer for 1 hr at appropriate hyb temp for LNA probe. Hyb and wash as above.
- Develop LNA probe first with NBT/BCIP. When colour is developed fix in 4% PFA + 0.2% glutaraldehyde o/n at 4°C. Wash 3 x 5mins in PBST. Wash for 40 mins – 1hr in 0.1M Glycine-HCl at pH2 (Sigma cat no G2879). Wash 4 x 5 mins in PBST, fix in 4% PFA + 0.2% glutaraldehyde o/n at 4°C. Then wash into MABT, block and apply anti-FITC-AP as above and develop 2nd colour. Other chromogenic reactions like Fast Red, new Fuchsin or Peroxidase coupled AB in combination with Benzidine may be taken into account when regarded necessary.



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