

WHOLE-MOUNT IN SITU HYBRIDIZATION ON VERTEBRATE EMBRYOS

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This *in situ* recipe is working well in a number of labs. It was developed for chick embryos but also works well on mice, *Xenopus* and fish embryos. Please use it freely and pass it to your colleagues. For publication, cite Henrique *et al. Nature* **375**: 787-790 (1995).

Modified from protocols of Ron Conlon (Mt. Sinai, Toronto), Phil Ingham (ICRF Oxford), David Wilkinson (NIMR, London) and Richard Harland (UC, Berkeley) with various other inputs. Note hybridization in much reduced salt concentration (i.e. at high stringency) and omission of RNase digestion. This gives specificity, sensitivity and low background. The recipe has worked with a 264bp RNA probe.

DISSECTIONS

1. Dissect out decidua and embryos in PBS
2. Transfer to dissecting medium (10% Fetal Calf Serum in DMEM + 25mM HEPES (pH 7.4) and remove extra-embryonic membranes. Puncture amnion to avoid probe trapping.
3. Rinse embryos in PBS.
4. Fix in 3ml 4% paraformaldehyde in PBS, 1-2h at room temp; or 4°C, 2h-o/n.
5. Wash twice in PTW.
6. Wash with 50% MeOH/PTW, then 100% MeOH twice; can store at this point at -20°C (for less than one month). PTW = PBS, 0.1% Tween-20.

PRETREATMENTS and HYBRIDIZATION

7. Rehydrate embryos through 75%, 50%, 25% MeOH/PTW (allowing embryos to settle), and washing twice with PTW.
8. Treat with 10µg/ml proteinase K in PTW: For mice: roughly no of mins = age in days. Best however to titrate your Prot K treatment.
9. Remove proteinase, rinse briefly (care !) with PTW, and post-fix for 20min in 4% paraformaldehyde + 0.1% Glutaraldehyde, in PTW.
10. Rinse and wash once with PTW. Transfer embryos to screw cap 2ml tubes.
11. Rinse once with 1:1 PTW/hybridization mix. Let embryos settle.

12. Rinse with 0.75 ml hybridization mix. Let embryos settle.
13. Replace with 0.75 ml hybridization mix and incubate horizontally =1h/70°C.
 - [Can store at -20°C before or after prehybridizing.]
14. Add 0.75 ml pre-warmed hybridization mix @ ~1µg/ml DIG-labelled RNA probe (possibly 0.1 µg/ml is enough). Immediately place at 70°C .
15. Incubate o/n horizontally at 70°C [Rock once after 20-30min].

Hybridization mix:

	Final conc.	
Formamide (Gibco/BRL Ultrapure)	50%	25ml
SSC (20x pH5.0)	1.3xSSC	3.25ml
EDTA (0.5M, pH8)	5mM	0.5ml
Yeast RNA (20mg/ml) (Sigma R-7125)	50µg/ml	125µl
Tween-20 (10%) (Sigma P-1379)	0.2%	1ml
CHAPS (10%) (Sigma C-3023)	0.5%	2.5ml
Heparin (50mg/ml) (Fisher H-19)	100µg/ml	100µl
H ₂ O (DEPC treated)		17.5ml
	Total	50ml

- Steps 5-10 are carried out on a roller, in 3 ml solution in 5ml round bottomed tubes. Thereafter, use 1 ml in a 2ml microtube, and rocking at room temperature unless otherwise stated. In this recipe, rinses are immediate, and washes are for 5min unless otherwise stated.
- 4% paraformaldehyde/PBS should be made on day of use.
- Hybridization mix can be stored at -20°C.
- A stock of glutaraldehyde, use high quality, is stored in aliquots at -20°C. Thaw out aliquot just before use.
- To make 200 ml of 20xSSC pH5.0
 - First make up 0.6M stocks of Citric Acid and Sodium Citrate (Na₃Citrate).
 - Mix 41 ml of 0.6M Citic Acid + 59 ml of 0.6M Sodium Citrate. May need to play around with proportion to end up with pH near 5.0 after adding salt.
 - Add NaCl to final of 3M in 200ml (35.06g). Finally make up to 200 ml with H₂O.

POST-HYBRIDIZATION WASHES

1. Rinse twice with prewarmed (70°C) hybridization mix.
 2. Wash 2 x 30 min/70°C with 1 ml prewarmed hyb mix.
 3. Wash 20 min/70°C with 1 ml prewarmed 1:1 hyb mix/MABT.
 4. Rinse 3x with 1 ml MABT (=100mM Maleic acid, 150mM NaCl, pH7,5, 0.5% Tween-20).
 5. Wash 2 x 30 min with 1 ml MABT
 6. Incubate 1h with 1 ml MABT + 2% Boehringer Blocking Reagent (BBR).
 7. Incubate =1h with 1 ml MABT + 2% BBR + 20% heat-treatedsheep serum.
 8. Incubate o/n at 4°C (or 4h at RT) in (fresh) 1ml MABT + 2% BBR + 20% serum + 1/2000 dilution of AP-anti-DIG antibody (Boehringer).
- Steps 2 and 3: incubate in oven with the tubes rolling. Steps 5-8: roll at room temperature.
 - After each 70°C wash, let embryos settle by incubating tube vertically at 70°C in a heating block, then change supernatants individually so samples don't cool. Keep wash solutions at 70°C in water-bath.
 - Sheep serum is heat-treated at 55-60°C, 30 min and stored in quick-frozen aliquots at -20°C.
 - Boehringer Blocking Reagent (#1096 176). Make 10% stocks in MAB (no Tween) heating to dissolve, then autoclave, aliquot and freeze. Works better than "embryo powder", and is easier and more reproducible to make. We have used MABT thereafter for consistency, but maleate does not buffer well, and alternative buffers probably substitute as well or better.

<u>5xMAB(T)=</u>	Maleic Acid (M-0375)	11.6g
	NaCl	8.7g
	Tween-20 (10%)	10 ml
	H ₂ O	~185ml
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	Total	200ml

Weigh, dissolve and pH maleic acid before adding other ingredients, this requires a lot of NaOH 10N. Autoclave before adding Tween-20

POST-ANTIBODY WASHES AND HISTOCHEMISTRY

1. Rinse 3 times with 1 ml MABT. Transfer to glass scintillation vial.
2. Wash 3x1h with 10 ml MABT, by rolling.
3. Wash 2x10 min with 10 ml NTMT.
4. Incubate with 1.5ml NTMT + 4.5 μ l/ml NBT (75mg/ml in Dimethyl Formamide) + 3.5 μ l/ml BCIP (X-phosphate; 50mg/ml in 70% DMF). Rock for first 20min, then incubate at room temp. for 30min to 3days. Optional: doubling the NBT and BCIP concentration speeds up staining and doubles the cost. (Optional: staining develops faster at 37°C but monitor very carefully).
5. When colour has developed to the desired extent, rinse 1x and wash =2x with PTW. Refix in 4% paraformaldehyde/0.1% glutaraldehyde/PTW for 2h (at room temp) or o/n (at 4°C). Rinse 1x and wash 2x 10min with PTW. Store at 4°C in PTW + 0.1% azide.

<u>NTMT:</u>	5M NaCl	1ml
	2M TrisHCl pH9.5	2.5ml
	2M MgCl ₂	1.25ml
	10% Tween-20	5ml
	H ₂ O	40.25ml
	Total	50ml

Make from stocks on day of use. NB, final Tween concentration is 1%.