

Whole mount in situ hybridization

This protocol is modified from E.De Rebertis (1997), Mechanism of Development.

Preparation of the probe

Ribo-probes are made using DIG RNA labelling mix (*Boehringer #1277 073*) following company's procedure.

Filter Detection

To detect both the quantity and quality of ribo-probes

- use a standard Dig-labelled RNA control (*Boehringer#1585 746*) to make serial dilutions : (1) 50ng/ul (2) 25ng/ul (3) 10ng/ul (4) 1ng/ul with 10xSSC.
- mix 1ul of sample with 1ul of 10xSSC .
- spot 1ul of each RNA control and sample on the nylon filter
- incubate filter for 30 min with 10ml of PBSw containing 1% blocking agent (*Boehringer#1096 176*), heat to dissolve in advance.
- dilute antibody AP (*Boehringer#1093 274*) 1:5000 in **TBST** containing 1% blocking agent. Incubate filter for 30 min with the solution.
- wash the filter 2x with **TBST**, 15min each.
- wash filter for 5 min with **NTM**.
- incubate filter in the dark with color reagent. (**NTM** + 4.5ul/ml NBT+3.5ul/ml BCIP)
- stop reaction with PBS and take picture.

Preparation of embryos

- make fresh 4% paraformaldehyde in PBS (powder heat to 60c with stirring until clear), store on ice.
- dissect embryo in cold PBS, briefly wash embryo with fresh PBS and transfer embryos to 15ml tube containing 4% paraformaldehyde.
- punch hole at brain cavities for embryo older than 9 dpc.
- when all embryos from same mother are dissected, renew the 15ml tube with fresh 4% paraformaldehyde.
- incubate embryos in 4% paraformaldehyde at 4c with rocking for 4 hrs or overnight for older embryos.
- second day, wash embryos 2x with PBSw.
- dehydrate embryos with methanol series (25%, 50%, 75%, 100% , 100% in PBS).
- store embryos at -20°C for later use.
- 4% paraformaldehyde.
- incubate embryos in 4% paraformaldehyde at 4c with rocking for 4 hrs or overnight for older embryos.
- second day, wash embryos 2x with PBSw.
- dehydrate embryos with methanol series (25%, 50%, 75%, 100% , 100% in PBSw).
- store embryos at -20°C for later use.

Hybridization

Day 1

- transfer embryos to 2.0ml screw cap vial.
- rehydrate the embryos through methanol series (75%, 50%, 25% and PBSw).
- wash embryos 3x with PBSw.
- replace buffer with 1ml of 7.5ug/ml proteinase K in PBSw.
incubate at RT for 7 min. for 7.5dpc embryo, 10 min. for 8.5dpc and 14 min. for 9.5dpc
(incubation times have to be optimized for different genes)
- stop digestion by washing in freshly prepared 2mg/ml glycine in PBSw.
- wash 3x with PBSw for 5 min. each.
- refix embryos in 1.5 ml of 4% paraformaldehyde+ 0.2% glutaraldehyde in PBSw for 15 min.
- wash 3x with PBSw for 5 min. each
- wash in 1.5 ml of 50% PBSw : 50% **hybridization solution** (5 min.)
- replace buffer with 900 ul of fresh **hybridization solution**.
- prehybridize embryos for 3 hrs at 65°C. oven with rotating
- heat 100ng to 250ng of the ribo-probe in 100ul of hybridization solution at 95°C for 5min.
- add heated probe to the embryos and incubate at 70°C oven for 10 min. with rotation.
- then overnight incubation at 70°C without rotating.

Day 2

- remove probe and add 800ul fresh **hybridization solution**, wash embryo for 5 min. at 70°C
- add 400ul of 2x SSC (pH4.5) to the wash and wash for 5 min at 70°C. , repeat the addition of 2xSSC and wash twice more.
- remove the mix and wash twice 30 each time in 2xSSC (pH7.0 : 0.1% CHAPS) at 70°C
- wash 30 min. in **MAB** at RT and twice 30 min each in **MAB** at 70°C
- wash twice 10 min in PBS at RT and 10 min in PBSw at RT.
- incubate embryos in 1 mL antibody buffer for 2 hrs at 4°C with rocking.
- at the same time, preabsorb antibody AP (*Boehringer#1093274*) with a dilution of 1 / 75000 in **antibody buffer** at 4°C with rocking for 2 hrs.
- replace buffer with preabsorbed antibody 1.5 mL and incubate at 4°C O/N with rocking.

Day 3

- rinse embryos with in PBSw.
- wash embryos 5x 45 min. each with 2.0 mL 0.1% BSA in PBSw at RT with rocking.
- wash twice 30 min. each with PBSw.
- wash embryos with **NTM** buffer 2x 10 min each.
- replace with staining solution (**NTM** buffer + 4.5ul/mL NBT + 3.5ul/mL BCIP + 2mM levamisole) and rock in the dark for 20 min. at RT
- check for staining, if background is low, stain embryos for overnight or longer at 4°C.
- after staining, dehydrate through methanol series for storage (-20°C)
- picture taking is done after rehydration.

Important Solution

10X **TBST** (100ml): 8g NaCl, 0.2g KCl, 3g Tris pH 7.6, 1% Tween-20

NTM (freshly made): 100 mM NaCl, 100 mM Tris pH 9.5, 50 mM MgCl₂

MAB : 100 mM maleic acid, 150 mM NaCl; pH 7.5

Hybridization solution: (50ml)

0.5g blocking agent (Boehringer)

25 ml formamide

12.5 ml 20X SSC, pH 7

heat to 65°C for 1 hr, then add

6 ml H₂O

5 ml yeast RNA

100 ul 50mg/ml heparin

250ul 20% Tween-20

500ul 10% CHAPS

500ul 0.5 M EDTA

filter the solution and store at -20°C

Antibody buffer :

1% blocking agent (Boehringer) in PBS (microwave dissolution)

0.1 % tween-20

10% goat serum (heat inactivated)

filter through 4.5 micron filters and cool on ice