Whole mount RNA in situ hybridization

Collection and storage of embryos

1. Dissect embryos in PBS, removing membranes as much as possible. (From E9.5 punture mylencephalon and make small hole in heart and telencephalon)
2. Fix in 10 ml of 4% paraformaldehyde at 4°C, ON with gentle rocking
3. Wash with PBT twice 5' at 4°C
4. Dehydrate through Methanol/PBT gradient (25%, 50%, 75% and twice 100%, 5' each at RT) (Can store at –20°C as long as months)

Day 1: Pretreatment of embryos

1. Rehydrate embryos through MetOH/PBT (75%, 50%, 25% and twice PBT, 5' each at RT).
2. Bleach with 6% (2 ml 30% + 8 ml PBT) hydrogen peroxide in PBT, 30 min at RT
3. Wash with PBT 3 x 5' at RT
4. Treat with 4 μg/ml (4-μl 10 mg/ml in 10-ml PBT) Proteinase K, 8 min at RT (10 μg/ml 15' for >E8.5 embryos)
5. Wash with fresh 2 mg/ml glycine solution (20 mg in 10 ml PBT), 10 min at RT
6. Wash with PBT 2X at RT
7. Refix with 4% paraformaldehyde in PBT 20 min at RT
8. Wash with PBT 2X 5 min at RT

Pre-hybridization and hybridization

9. Exchange 3X with hybridization buffer.
10. Add 500 μl of hybridization buffer per tube, 70°C for 2~3 hours
11. Add 1-2 μl probe and hybridize at 70°C ON

Solutions

Hybridization Buffer (10 ml)

50% Formamide	5 ml
5X SSC	2.5 ml 20X
50 μg/ml tRNA	50 μl
1% SDS	500 μl 20%
50 μg/ml Herparin	10 μl
H_2O	1.94 ml

PBT

PBS + 0.1% Tween 20 (0.5 ml Tween 20 in 500 ml of PBS)

Day 2: Post-hybridization wash

1. Wash with Solution I (prewarmed) 2 X 30 min at 70°C
2. Wash with Solution I: Solution II (1:1) for 10 min at 70°C
3. Wash with Solution II for 3 X 5 min at RT
4. Incubate with 100 μg/ml RNase A (100-μl 10mg/ml in 10 ml of Solution II) for 30 min at 37°C
5. Wash with Solution II 5 min at RT then Solution III 5 min at RT
6. Wash with Solution III for 2X30 min at 65°C (prepare MAB+2% BMB during these steps)

Antibody Reaction

7. Wash with MAB buffer 3 X 5 min at RT
8. Block with MAB+2% BMB with 10% goat serum. Nutate for 3h at RT (prepare BAS during this step)
9. Replace with BAS (500 ml/tube). Incubate O/N at 4°C

Solutions

Solution I
25 ml formamide
10 ml 20X SSC (pH 4.5)
2.5 ml 20% SDS
Bring up to 50 ml with water

Solution II
20 ml 5M NaCl
2 ml 1M Tris, pH 7.5
2 ml 10% Tween 20
Bring up to 200 ml with water

Solution III
20 ml formamide
4 ml 20X SSC
Bring up to 40 ml with water

Heat Inactivated Goate Serum
Place bottle at 65°C for 30 min. Aliquot and store at –20°C.

2 X MAB Buffer (1L)
200 mM Maleic Acid 23.22g
300mM NaCl (pH7.5) 17.53g
Titer pH to 7.5 using 10N NaOH
For 50 ml 1X MAB: just prior to use,
Add 25mg of levamisole and 500 μl of 10% Tween

MAB+2% BMB
0.2g of BMB (Blocking reagent 1096176 BM)
10ml of MAB+Lev+Tween
Heat at 60C to dissolve
Use 2ml for BAS
Use remainder for blocking step

Preparing Blocked Antibody Solution (BAS)
Pour a few flakes of embryo powder into 500 μl MAB+2%BMB and incubate at 70°C for 30min.
Vortex for 10s and cool on ice.
Add 5 μl of goat serum and 1μl anti-DIG AP FAB fragment (MB 1093274) antibody. Nutate at 4°C for 1h.
Spin at 4C for 10min.
Dilute supernatant to 2ml with MAB+2%BMB with 1% goat serum
This is for 4 tubes at 500 μl/tube (1:2000final dilution).
To scale up: can pre-absorb up to 4 μl of antibody in 500 μl MAB+2%BMB.

Day 3: Post-antibody washes

1. Wash with MAB buffer 3 X 5 min at RT
2. Wash 10~12 X 30 min at RT on a nutator (can leave final wash O/N on nutator at 4°C)

Color reaction

3. Wash with NTMT 3 X 10 min at RT.
4. Incubate with BM purple (BM 1442074) +2.5mg/5ml (2mM) levamisole wrapped in foil in the dark at RT
5. When reaction is complete, wash in PBT 3C and refix in 4% PFA for 1hr to O/N.
6. Transfer embryos to 50% then 70% glycerol.

Solutions

NTMT
10 ml of 1M Tris pH 9.5
5 ml of 1M MgCl
2 ml of 5M NaCl
50 mg Levelmisole (0.1g levelmisole in 0.45ml water, and add 0.25 ml to NTMT)
1 ml of 10% Tween
Bring up to 100 ml with water