Whole mount in situ hybridization to study gene expression during mouse development

1. Riboprobe preparation

Set up a restriction reaction to linearize the plasmid DNA. Check for complete digestion, then incubate with 200 µg/ml PK in 0.5% SDS at 37°C for 60 min. Extract with phenol/chloroform, and recover by ethanol precipitation. Wash the pelleted DNA with 70% ethanol; redissolve in Rnase-free TE buffer at a concentration 1mg/ml.

Set up a labeling reaction according to the RNA polymerase instructions in total volume 20 µl. Incubate @ at 37°C for 60 min. Add 2 µl of 0.5M EDTA (pH=8.0) and 4 µl of 4 M LiCl, mix, then add 120 µl of 100 ethanol and put at - 20°C for a few hours. Pellet the labeled RNA at max speed in a microfuge at 4°C for 30 min. Wash the pellet with 70% ethanol, dissolve in 20 µl of Rnase-free water. Examine 1 µl on 1% agarose gel.

2.WMISH

Day1

For each basket of embryos to be hybridized, fill 12 successive wells of 24 well plate with 2 ml of the following solutions: 1 well- 75% MeOH in PBT, 1- well 50% MeOH in PBT, 1- well 25% MeOH in PBT, 2- PBT, 1-PK (see notes), 1- PBT, 1-PGF postfix, 2- PBT, 1- 1:1 hyb buffer:PBT, 1- hyb buffetr. Move the basket through the well as described in the table below.

75% MeOH, 5 min	50% MeOH, 5min	75% MeOH, 5 min	PBT, rinse	PBT, 5 min	PK, See notes
PBT, rinse	PGF postfix, 20 min	PBT, 5 min	PBT, 5 min	Hyb:PBT, 5min	Hyb, 5 min

Remove the basket from the final well and place into a scintcilation vial with 3 ml of Hyb byffer. Cap the vial and place at 70°C to pre-hybridize for 1hour.

Take 1ml of the Hyb buffer from the vial into a microfuge tube containing a probe (1-2 µg) from step 1, mix well and return into the vial, leave at 70°C overnight.

Day2

Following overnight hybridization, remove probe solution. Use a Pasteur pipette placed down between the basket and the side of the vial to aspirate out the buffer.

- Rinse twice with 2 ml of Hyb buffer (prewarmed to 70^{o}C).
- Wash twice for 30 min each time at 70^{o}C with 2 ml of Hyb buffer (prewarmed to 70^{o}C).
- Wash once for 10 min at 55^{o}C with a 1:1 mix of Hyb buffer and TBST (prewarmed to 55^{o}C).
- Take the basket trough the wells of 24 well plate with gentle rocking as described below:

TBST, Rinse	TBST, Rinse	TBST, 15 min	Buffer A, 1 hour	Buffer B, 1 hour

- Remove the basket from the last well and place into a scintillation vial with 3 ml of Buffer B containing 1.5 µl of AP coupled anti-DIG antibody (Roche), incubate at 4^{o}C overnight with gentle rocking.

Day3

Following overnight hybridization, wash the embryos with gentle rocking

- 3 times for 1 hour each in 100 ml of TBST

Remove the embryos from the basket and place them into a well of 12 well plate, remove the TBST, rinse the embryos in the well very briefly with water and add BM Purple AP substrate (Roche). Stain in the dark until sufficient color develops. Terminate the reaction by removing the substrate and adding PBT, rinse and wash in PBT a couple of times. At this point embryos can be stored at 4^{o}C or prepared for embedding.

Buffers and solutions

PBT

Phosphate-buffered saline without $MgCl_2$ and $CaCL_2$ supplemented with 0.1% Tween

Stock	50 ml	100 ml
10 X PBS	5 ml	10 ml
10% Tween	0.5 ml	1 ml

TBST

1X TBS with 0.1 Tween

Stock	500 ml	1000 ml
10 X TBS	50 ml	100 ml
10% Tween	5 ml	10 ml
H_2O	445 ml	890 ml

MeOH in PBT

Methanol mixed with PBT to give 75, 50 or 25%

PK

Stock solution is 0.1g/ml. Working concentration depends on the stage of embryos in the experiment. Time of incubation depends upon the stage as well. As a guideline, for E 8.5, working concentration is 20 µg/ml and incubation time 10 min.

PGF postfix

4% Paraformaldehyde, 0.025% glutaraldehyde in PBS.

	2 ml	4 ml	8 ml	12 ml
10 X PBS	0.2ml	0.4 ml	0.8 ml	1.2 ml
16 %PFA	0.5 ml	1 ml	2 ml	3 ml
8% GA	6.25 µl	12.5 µl	25 µl	37.5 µl
H_2O	1.3 ml	2.6 ml	5.2 ml	10.4 ml

Hyb buffer (make fresh every time)

50% Formamide
0.75M NaCl
1X PE
100 µg/ml tRNA
0.1% BSA
1% SDS
0.1% Tween
in DEPC water.

Final con	Stock	100 ml	50 ml	25 ml
50% FA	100 %	50 ml	25 ml	12.5 ml
0.75 M NaCL	5M	15 ml	7.5 ml	3.75 ml
1XPE	10X	10 ml	5 ml	2.5 ml
0.1% BSA	100 mg/ml (10%)	1 ml	0.5 ml	0.25 ml
1% SDS	20 %	5 ml	2.5 ml	1.25 ml
0.1 % Tween	10 %	1 ml	0.5 ml	0.25 ml
100 µg/ml tRNA	10 mg/ml	1 ml	0.5 ml	0.25 ml
H_2O		17 ml	8.5 ml	4.25 ml

Buffer A

2% BBR in TBST

	4ml	6 ml	8 ml	10 ml
1 X TBST	3.2	4.8 ml	6.4 ml	8 ml
10% BBR	0.8 ml	1.2 ml	1.6 ml	2 ml

Buffer B

2% BBR, 20% goat serum in TBST

	4ml	6 ml	8 ml	10 ml
1 X TBST	2.4 ml	3.6 ml	4.8 ml	6 ml
10% BBR	0.8 ml	1.2 ml	1.6 ml	2 ml
100% Goat serum	0.8 ml	1.2 ml	1.6 ml	2 ml

BBR

10% Blocking Reagent (Roche) in TBS, store frozen in 10 ml aliquots.

1O X PE

100 mM PIPES and 10 mM EDTA. Store at 4 ^{o}C.

1M PIPES

Make 1M PIPES and store in 10 ml aliquots frozen.