# Protocol for Whole-mount *in situ* Hybridization

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Original methods derived from J. Rossant Lab.

(Conlon, R. A. and Rossant, J. 1992. *Development* **116**, 357-368.)

Modified according to the method of A. McMahon Lab on Oct. 9, 1993.

## I. Tissue Preparation

1. Dissect embryos free from decidua in PBS. For embryos older than 8.5 dpc, yolk sac and amnion should be removed.

2. Fix embryos in an adequate amount of **fresh** fixative (4% paraformaldehyde in PBS) with rocking for 2-12 hr at 4°C.

3. Wash three times on ice with PBS containing 0.1% Tween-20 (PBT). The embryos may be stored at -20°C after dehydration through ascending series of methanol. Embryos in methanol survive a couple of weeks of storage without much degeneration.

4. Bleach the embryos in 5:1 methanol/30% hydrogen peroxide for 5-6 hrs at R/T, followed by several washes in methanol. The embryos can be stored again in methanol at -20°C. To resume the procedure, rehydrate the embryos through graded series of methanol/PBT (75-50-25%) at R/T. Then wash through several changes of PBT.

## II. Pretreatment & Hybridization

1. Treat samples with 20 µg/ml proteinase K in PBT for 5 min at R/T. Don't shake embryos in solution.

2. Wash gently twice with **freshly prepared** 2 mg/ml glycine in PBT. Wash twice with PBT.

3. Refix the embryos in **fresh** 0.2% glutaraldehyde/4% paraformaldehyde in PBS at R/T for 20 min.

4. Wash embryos through three changes of PBT. Treat with **freshly prepared** 0.1% sodium borohydride in PBT for 20 min. DO NOT CAP THE TUBES TIGHTLY: Borohydride produce copious amounts of hydrogen gas.

5. Wash with three changes of PBT. Remove air bubbles under a dissection microscope, and transfer them into tubes for hybridization.

6. Wash twice with hybridization buffer. The embryos become translucent in solutions containing 50% formamide. Incubate for at least 1 hr at 63°C.

7. Replace hybridization buffer, and add briefly heated (at 65°C for 5 min) probe solution to 0.2-2 µg/ml. Hybridize overnight at 63°C.

## III. Washing & Probe Detection

1. Wash briefly once with Washing buffer 1. Wash twice with Washing buffer 1 for 30 min each at 63°C with rocking.

2. Wash twice with Washing buffer 1.5 for 30 min each at 50°C with rocking.

3. Wash once with RNase buffer. Treat with 100 µg/ml RNase A ( and also with 100 U/ml RNase T1) in RNase buffer for 1 hr at 37°C with rocking. Wash once with RNase buffer.

4. Wash with Washing buffer 2 for 30 min at 50°C.

5. Wash with Washing buffer 3 for 30 min at 50°C (45°C for AT-rich probes). At this point, the embryo powder for preabsorption of the antibody should be inactivated by heating a few milligrams of powder in 1 ml of TBST to 70°C for 30 min, then vortexed for 10 min prior to cooling on ice.

6. Wash twice with Washing buffer 4, then place embryos in a heating block at 70°C for 20 min.

7. Rock embryos in TBST containing 2mM levamisole (0.5 mg/ml **freshly added**) and 10% heat-inactivated goat serum for at least 1 hr at R/T. At this time, preabsorb the antibody by diluting the antibody to 1/2000-1/5000 in cold TBST containing 2mM levamisole, 1% freshly heat-inactivated goat serum and the heat-inactivated embryo powder.

Rock the tube for 30 min at 4°C, Centrifuge the mixture at 10,000xG for 10 min at 4°C. The preabsorbed antibody is in the supernatant.

8. Incubate the embryos with the preabsorbed antibody overnight at 4°C with rocking.

9. Wash three times with TBST containing 2mM levamisole (**fresh**), then wash 5 or 6 times, one houre each, at R/T with rocking in the same buffer.

10. Wash twice with freshly prepared NTMT containing **fresh** 2mM levamisole for 20 min each at R/T with roking.

11. Start reaction with color reagents (4.5 µl/ml NBT, 3.5 µl/ml BCIP and 2 mM levamisole in NTMT). Rock the tubes for the first 5 min of reaction, then transfer to a small dish, and allow to develop **without** rocking.

12. Stop color reaction by washing twice with NTMT, then extensive washing with 3 changes of PBT(pH5.5) containing 1mM EDTA to prevent further alkaline phosphatase activity. Postfix the embryos with 4% paraformaldehyde/0.1% glutaraldehyde in PBS for 1 hr, wash several times in PBT and store at 4°C, or proceed to the next step.

13. Wash the embryos through PBT, then dehydrate through 30, 50, 70 and two changes of 100% methanol. Rehydrate by going down the series of to PBT. Clear the embryos by passing the embryos into 1:1 glycerol/PBT, and then into 4:1 glycerol/PBT for 1 hr each with rocking.

# Appendix

## I. Solutions

1. Hybridization Buffer

50% formamide
0.75M NaCl
1X PE
100 μg/ml tRNA
0.05% heparin
1% SDS

2. Washing Buffers

Wash 1 300 mM NaCl
1x PE
1% SDS

Wash 1.5 50mM NaCl
1x PE
0.1% SDS

Wash 2 50% formamide
300mM NaCl
1x PE
1% SDS

Wash 3 50% formamide
150mM NaCl
1x PE
0.1% Tween-20

Wash 4 500mM NaCl
1x PE
0.1% Tween-20

3. NTMT 100mM Tris pH 9.5
   100mM NaCl
   50mM $MgCl_2$
   0.1% Tween-20

4. RNase Buffer
   10 mM Tris-Cl (pH. 8.0)
   1 mM EDTA
   500 mM NaCl
   0.1% Tween-20

## II. Probe Preparation

1. Mix the following reagents;

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|---|---|---|
| Linear template (1 μg/μl) | | 1 μl |
| DDW | 3.5 μl | |
| 5X Transcription buffer | 4 μl | |
| 10mM ATP, GTP, CTP | | 2 μl each |
| 10mM UTP | | 1.2 μl |
| 10mM Dig-UTP | | 0.8 μl |
| 100mM DTT | 2 μl | |
| RNase inhibitor (40 U/μl) | | 0.5 μl |
| RNA polymerase (40 U/μl) | | 1 μl |
| Total | | 20 μl |

2. Incubate for 2 hr at 37°C

3. Add 1 μl of 1 mg/ml DNase I (RNase free), and incubate at 37°C for 10 min.

4. Add 80 ml of stop solution ( 20mM Tris pH7.5, 20mM EDTA, 100mM NaCl, 1% SDS).

5. Spin through Sephadex G-50 column (DEPC treated, washed and autoclaved in 0.3M NaOAc/0.1% SDS).

6. Add 2 vol. of EtOH, chill at -80°C, and precipitate transcripts.

7. Dissolve in DEPC-water ( or TE 7.6) and heat for a second at 75°C, and then add formamide to 50%, stored at -80°C.

## III. Prepartion of Embryo Acetone Powder

1. Collect 13.5 dpc. embryos in ice-cold HCMF.
2. Homogenize embryos.
3. Add four volume of ice-cold acetone and mix vigorously. Keep on ice for 30 min with occasional vigorous mixing.
4. Centrifuge 10,000 XG for 10 min and discard the sup.
5. Resuspend the pellet in cold acetone and mix vigorously. Sit on ice for 10 min and centrifuge again.
6. Transfer the pellet on a clean filter paper, spread the precipitate and allow air-dry at R/T.
7. Store the dried powder in an air-tight vial at 4°C.

## IV. Stock Solutions

5M NaCl
5M LiCl
1M $MgCl_2$
0.5M EDTA
10 mg/ml yeast tRNA
10% SDS
10% Tween-20
5% heparin
2M Tris pH 9.5
2 mg/ml Proteinase K
20 mg/ml RNase A
10X PE (100mM PIPES pH 6.8, 1mM EDTA)
10X PBS (80 g NaCl, 2 g KCl, 11.5 g Na2HPO4, 2 g KH2PO4/ 1 L)
75 mg/ml NBT (nitroblue tetrazolium salt) in 70% dimethylformamide

50 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate, **toluidine salt**) in 100% dimethylformamide