

Whole Mount in situ-Protocol 1

1. Cleanliness throughout the procedure is important. Dirt accumulates and tends to stick to the embryos. Dissect 6.5 to 10.5 day embryos free from decidua in DEPC-treated PBS, and reflect or remove the yolk sac and amnion from 8.5 and 10.5's. Embryos with closed anterior neuropores on day 9 and 10 should have the brain punctured to allow flow of solutions in and out (brains can be punctured at this point, or later when the embryos are in hybridization buffer). The procedure also works for 12.5-13.5d lungs.
2. Fix embryos in 10 ml of fresh fixative (4% paraformaldehyde in PBS) at room temperature. Invert the tube several times and transfer to ice. Replace fix with ice-cold fix. Rock or rotate the tube gently for two hours at 4°C.
3. Wash three times on ice with 10 ml of PBS containing 0.1% Tween-20 (PBT). ("Wash" means to invert the tube several times and let the embryos settle to the bottom) The embryos may be stored at this point. To store, transfer the embryos into 100% methanol and store at -20 °C. 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 hours at room temperature, followed by several washes in methanol. The embryos can be stored in methanol at -20 °C. To resume the procedure, rehydrate the embryos through a graded series of methanol/PBT (75, 50 and 25%) at room temperature. Then wash through several changes of PBT.
5. Transfer the embryos to a 2 ml screw-cap tube. Treat with 20 µg/ml proteinase K in PBT for 5 to 10 minutes at 37°C with rocking. The length of the protease treatment may need to be altered for a particular batch of protease.
6. Wash twice with freshly prepared 2 mg/ml glycine in PBT. Wash twice with PBT.
7. Refix the embryos in fresh 0.2% glutaraldehyde/4% paraformaldehyde in PBS at room temperature for 20 minutes with rocking.
8. Wash the embryos through three changes of PBT. Treat with freshly prepared 0.1% sodium borohydride in PBT for 20 minutes in upright tubes. DO NOT CAP THE TUBES TIGHTLY--borohydride produces copious amounts of hydrogen gas.
9. Wash with three changes of PBT.10.Wash twice with hybridization buffer. The embryos become translucent in solutions containing 50% formamide, therefore care must be taken in transferring and washing the embryos. Prehybridize for at least 1 hour at 63°C with rocking.
11. Replace hybridization buffer with enough buffer to almost fill the tube, and add 10 µl of probe prepared as described below. Hybridize overnight at 63°C with rocking.

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12. Wash once with Wash 1. Wash twice with Wash 1 for 30 minutes each at 63 °C with rocking.
13. Wash twice with Wash 1.5 for 30 minutes each at 50 °C with rocking.
14. Wash once with RNase buffer. Treat with 100 µg/ml RNase A and 100 U/ml RNase T1 in RNase buffer for 60 minutes at 37 °C with rocking. Wash once with RNase buffer.
15. Wash with Wash 2 for 30 minutes at 50 °C with rocking. At this point prepare a solution of 1% BMB Blocking Reagent in PBT by heating to 70 °C and stirring for 30 minutes. Cool the solution. The embryo powder for preabsorption of the antibody should be inactivated by heating a few milligrams of powder in 1 ml of PBT to 70 °C for 30 minutes.
16. Wash embryos with Wash 3 for 30 minutes at 50 °C (45 °C for AT-rich probes) with rocking.
17. Wash twice with Wash 4, then place embryos in a water bath at 70 °C for 20 minutes.
18. Rock embryos in PBT containing 2 mM levamisole (0.5 mg/ ml freshly added) and 1% BMB Blocking reagent for at least 1 hour at room temperature. At this time: preabsorb the antibody by diluting the antibody to 1/5000 in cold PBT containing 2 mM levamisole, 1% Blocking Reagent and the heat-inactivated embryo powder. Rock the tube for 30 minutes at 4 °C. Centrifuge the mixture at 10,000Xg for 10 minutes at 4 C. The preabsorbed antibody is in the supernatant.
19. Incubate the embryos with the preabsorbed antibody overnight at 4 °C with rocking.
20. Wash three times with PBT containing 2 mM levamisole (fresh), then wash 5 or 6 times, one hour each, at room temperature with rocking in the same buffer. Wash the 9-10 day embryos in a 10 ml volumes.
21. Wash twice with freshly prepared NTMT containing fresh 2 mM levamisole for 20 minutes each at room temperature with rocking.
22. Start color 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 minutes of the reaction, then stand the tubes in a rack for the remainder. The purple reaction product should be visible within 5 minutes for prevalent messages and can be complete within 20 minutes. For most messages the color reaction can proceed overnight at room temperature. Protect from light. Be patient when staining the embryos: if the embryos are transferred back and forth between tube and dish, or otherwise excessively disturbed, a precipitate forms.
23. Stop color reaction with 3 changes of PBT and store at 4 °C, or wash with three changes of TBST and proceed to next step.
24. Wash the embryos through TBST, then dehydrate through 30%, 50%, 70% and two changes of 100% methanol. The dehydration intensifies the pink-to-

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purple reaction products to dark blue. Rehydrate by going down the series to TBST. Clear the embryos by passing the embryos into 1:1 glycerol/CMFET, and then into 4:1 glycerol/CMFET for 1 hour each with rocking. To prevent bacterial growth, the glycerol solutions should contain 0.02% sodium azide.

Preparing embryo acetone powder

1. Homogenize 13 1/2 day embryos in a minimum of calcium-magnesium-free PBS on ice.
2. Add four volumes of cold acetone and mix vigorously. Keep on ice for 30 min with occasional vigorous mixing. Collect the precipitate by centrifugation at 10,000Xg for 10 minutes. Remove and discard the supernatant.
3. Resuspend the pellet with cold acetone and mix vigorously. Allow to sit on ice for 10 minutes. Spin at 10,000Xg for 10 minutes. Transfer the pellet to a clean piece of filter paper, spread the precipitate and allow to air-dry at room temperature. As it dries, continue to spread and disperse the pellet. After the powder is dry, transfer it to an airtight container and store at 4°C.

Probe Preparation

BMB has a protocol which works reasonably well for T3, T7 and SP6 polymerases. Note that the way the probe is handled after synthesis is altered because of the difference in solubility of the steroid-modified RNA.

Linear template (1 µg/µl)	1 µl
Water	5.5 µl
10X Transcription Buffer*	2 µl
10 mM ATP	2 µl
10 mM GTP	2 µl
10 mM CTP	2 µl
10 mM UTP	1.2 µl
10 mM dig-UTP	0.8 µl
250 mM DTT	1 µl
Placental RNase inhibitor	0.5 µl
RNA polymerase (20 U/µl)	<u>2 µl</u>
total volume	20 µl

*400 mM Tris-HCl pH 8.0, 60 mM MgCl₂, 20 mM spermidine, 100 mM NaCl

Incubate for 2 hours at 37°C. Then add 2 µl 0.2 M EDTA, 2.5 µl 4 M LiCl and 75 µl ethanol, and ppt at -20°C for 2 hours. Dissolve the pellet in 22.5 µl of water then add 2.5 µl 4 M LiCl and 75 µl EtOH and ppt at -20°C. Take up in 200 µl of hybridization buffer at 50°C for 10 minutes.

The labeling reaction should result in 5-20 µg of labeled RNA (at a concentration of 0.05-0.2 µg/µl in the 200 µl of hyb). To ascertain whether this was achieved, spot 2 µl of 1/100 and 1/1000 dilutions in 10X SSC on a nylon filter along with the same dilutions of dig-labeled DNA standards (0.1 µg/µl of labeled DNA obtainable from BMB). Detect with antibody directly as described below. Some SP6-transcribed templates have not produced reasonable amounts of transcripts in my hands. When the inserts were recloned and transcribed by T7 or T3 they worked fine. This appears to be a recurring

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phenomenon with many templates. Reagent kits designed to massive amounts of RNA in vitro do not work with digoxigenin, despite manufacturer's claims-- save your money. I have not found any improvement in signal or signal/noise using probes whose size was reduced by alkaline hydrolysis.

Filter Detection

All the following incubations are performed at room temperature. Except for the color reaction, all incubations require shaking or mixing.

1. Incubate filter for 30 min with about 20 ml of PBT containing 1% BMB blocking reagent (prepare the solution 1 h in advance by dissolving at 50-70°C. The solution remains cloudy.).
2. Dilute antibody-conjugate 1/5000 in TBST containing 1% blocking reagent. Incubate filters for 30 min with the diluted antibody solution.
3. Wash the filters 2 times for 15 min with TBST.
4. Equilibrate membrane for 2 min with NTM (100 mM NaCl, 100 mM Tris pH 9.5, 50 mM MgCl₂).
5. Incubate filter in the dark with color reaction mixture (NTM containing 4.5 µl/ml NBT and 3.5 µl/ml BCIP).
6. Stop reaction with CMFET, and store in CMFET.

Supplies and Solutions

Aldehydes are EM grade from Polysciences. Glutaraldehyde is obtained as an 8% solution in sealed 10 ml ampoules. After opening an ampoule, the remainder is aliquoted and stored at -20°C. Each aliquot is used only once. Make 20% paraformaldehyde fresh before use: Heat 17 ml of water on a hot plate with stirring. Add 1 drop of 10 N NaOH, then 4 g of paraformaldehyde and stir until dissolved. Make up to 20 ml. Antidigoxigenin-alkaline phosphatase conjugate (BMB)Digoxigenin-UTP (BMB)Blocking Reagent (BMB)

Stock Solutions

Solutions marked with a star (*) should be RNase-free. For RNase-free solutions: RNA/protein-free solutions should be autoclaved with 0.1% diethylpyrocarbonate (DEPC); RNA/protein-containing solutions should be made in DEPC-treated water. Other solutions should be sterile. Generally I consider it sufficient to keep steps up to and including hybridization RNase-free, and subsequent steps, merely sterile.

5 M NaCl
20% SDS 1 M MgCl₂
10 mg/ml tRNA*
10% BSA*
5% heparin*1 M Tris pH 9.5
10% Tween-20*
4 M LiCl*0.2 M EDTA*
10 mg/ml RNase A
20 mg/ml proteinase K*
75 mg/ml NBT (nitroblue tetrazolium salt) in 70% dimethylformamide

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50 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidine salt) in 100% dimethylformamide
10X PBS* (100 ml) -8 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄, 0.2 g KH₂PO₄
10X PE* (100 ml) (1X PE is 10 mM PIPES pH 6.8, 1 mM EDTA)

Hybridization Buffer*:

to make 10 ml		Stock
50% formamide	5 ml	formamide
0.75 M NaCl	1.5 ml	5 M NaCl1X
PE	1 ml	10X PE
100 µg/ml tRNA	0.1 ml	10 mg/ml tRNA
0.05% heparin	0.1 ml	5% heparin
0.1% BSA	0.1 ml	10% BSA
1% SDS	0.5 ml	20% SDS

Wash 1	Wash 1.5
300 mM NaCl	50 mM NaCl
1X PE	1X PE
1% SDS	0.1% SDS

RNase Buffer	Wash 2
0.5 M NaCl	50% formamide
10 mM PIPES pH 7.2	300 mM NaCl
0.1% Tween-20	1X PE
	1% SDS

Wash 3	Wash 4
50% formamide	500 mM NaCl
150 mM NaCl	1X PE
1X PE	0.1% Tween-20
0.1% Tween-20	

10X TBS (100 ml) 8 g NaCl, 0.2 g KCl, 3 g Tris pH 7.6 (TBST is TBS with 0.1% Tween-20)

NTMT (100 mM NaCl, 100 mM Tris pH 9.5, 50 mM MgCl₂, 0.1% Tween-20)NTM is same without Tween-20. Best if made fresh from stocks because ppt tends to form on standing.

CMFET (100 ml) 0.8 g NaCl, 0.02 g KCl, 0.115 g Na₂HPO₄ anh., 0.02 g KH₂PO₄ anh., 0.02 g EDTA, 0.1% Tween-20.

NOTES

The three most frequent sources of problems are the probe, the protease treatment and inactivation of the endogenous alkaline phosphatase of the embryo. 1) Sufficient probe must be present to drive the hybridization. However, if yields of the transcription reaction are low, increasing the proportion of the reaction added to the hybridization will result in high background. It is better to repeat the reaction and hope for better yield, or try a different probe. 2) The protease treatment must be right on for good results--too much digestion results in poor morphology and low signal; too little digestion results in high background. You must determine the conditions that work for you personally, and then be assiduous in

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duplicating those conditions exactly (the way that you bring your solutions to temperature, the length of treatment, the way you wash out the protease, etc.). 3) The level of alkaline phosphatase in the embryos is astounding, and the resistance to inactivation mind-boggling--take my word for it. The heat inactivation of alkaline phosphatase must be at 70 °C and for at least 20 minutes, or it will not be inactivated--background will be horrible. You have been warned.

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