

Whole-Mount *in Situ* Hybridization to Mouse Embryos

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The mouse is well-established as the major animal model for the study of mammalian development. Rapid progress in large-scale cDNA and also genomic sequencing projects is identifying new mouse genes at an unprecedented rate. As a first step toward understanding the function of these novel genes, it is important to determine their developmental expression pattern. Here we provide a reliable, sensitive method for whole-mount *in situ* hybridization using the mouse embryo.

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This method has been adapted from an earlier protocol (1) that has proven to provide extremely consistent results with mouse embryos. We have introduced some minor changes to the protocol which allow greater reproducibility while minimizing the time involved in handling the samples. For example, the proteinase K treatment is performed at 37°C, rather than at a potentially variable room temperature (2, 3). In addition, the length of posthybridization washes is increased, and the frequency of postantibody washes is increased, which gives reproducible results with low background.

MATERIALS

Stock Solutions

Diethyl pyrocarbonate (DEPC) is used to inactivate RNases. Solutions that may be DEPC-treated

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directly are marked with a superscript dagger. In these cases, add 1 μ l of DEPC/ml of solution, directly to the bottle, shake vigorously, let sit overnight, then autoclave. Solutions to make up with autoclaved DEPC-H₂O are marked with a superscript asterisk.

1. 10× PBS[†] (100 ml): 8 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄, 0.2 g KH₂PO₄

2. PBT[†]: PBS+ 0.1% Tween 20.

3. 8% Glutaraldehyde, electron microscopy grade (Polysciences): Comes in ampoules and is stored in aliquots at –20°C.

4. 20% Paraformaldehyde in PBS: Add 2 g electron microscopy-grade paraformaldehyde (Polysciences) to 10 ml PBS and 10 μ l 10 N NaOH. Heat with stirring to dissolve. Store at –20°C in 1-ml aliquots for up to 1 month.

5. Proteinase K stock*: 20 mg/ml in H₂O stored in 100- μ l aliquots at –20°C.

6. MABT: 100 mM maleic acid, 150 mM NaCl, 0.1% Tween 20 Adjust pH to 7.5 with solid NaOH. Do not treat with DEPC.

7. MABT + 2% BBR (Boehringer Blocking Reagent, Catalog No. 1-096-176). Make fresh on day of use. Heat with stirring to dissolve, then adjust pH to 7.5.

8. Heat inactivate normal goat serum at 65°C for 1 h. Higher temperatures or longer incubation may cause the serum to denature and solidify. Store at –20°C in 2-ml aliquots.

9. Nitroblue tetrazolium salt (NBT) stock: 75 mg/ml in 70% dimethylformamide.

10. 5-Bromo-4-chloro-3-indolylphosphate, toluidine salt (BCIP) stock: 50 mg/ml in 100% dimethylformamide.

Plasticware and Equipment

1. Flat-bottom 2-ml tubes with O-ring caps (Outpatient Services, No. 2059).

2. An oven for hybridization and washing of probe, such as the Autoblot microhybridization oven from Bellco.

3. An apparatus for rocking the embryos in the oven. For example, foam racks from Fisher (Catalog No. 05-664-15A) can be cut to fit the rotator inside the hybridization oven.

4. Nutator (Adams) or other apparatus for gently rocking samples throughout procedure.

5. Petri dishes (Falcon Catalog Nos. 1008, 1007) for dissection.

6. Watchmakers forceps (Fine Science Tools, Catalog No. 11252-30) for dissection.

7. Dissecting microscope.

METHODS

For the following protocol, the direction "rinse" means to add solution, invert the tube once, and then remove the solution when the embryos have settled to the bottom of the tube. The direction "wash" means 5 min with rocking, unless otherwise specified. All washes and incubations are done with rocking unless otherwise specified.

Dissections and Fixation

1. Dissect embryos in ice-cold PBS (4, 5).

2. Use a blue pipet tip, with the end cut off, to transfer one litter of embryos in a small volume of PBS to a 15-ml conical tube of ice-cold 4% paraformaldehyde in PBS made fresh from stocks. Gently invert the tube several times, then replace with fresh fix and incubate for 2 h at 4°C.

3. Wash three times in ice-cold PBT.

4. Wash three times in 100% methanol. Store in methanol at -20°C.

Synthesis and Yield of Probe

We find it most convenient to synthesize digoxigenin-labeled RNA probe using the method provided

by the supplier (Boehringer Mannheim). Additional details on probe preparation, in general, and the synthesis of nonradiolabeled digoxigenin or fluorescein probes, in particular, are provided in the article by Abraham. To estimate the amount of probe synthesized it may be convenient to compare it with a digoxigenin-labeled RNA standard (Boehringer Mannheim, Catalog No. 1585746). We find the following procedure to work well.

1. Spot 2 μ l of 1:10, 1:100, and 1:1000 dilutions of both the newly synthesized probe and the reference standard on a nylon hybridization membrane. Allow to dry completely.

2. Block the membrane with 2% BBR in MABT for 15 min with rocking. For a 1 \times 2-in. square of membrane, incubations can be carried out in a 15-ml conical tube. Do not allow different pieces of membrane to overlap.

3. Incubate with a 1:5000 dilution of anti-digoxigenin-AP antibody in 2% BBR in MABT for 30 min with rocking.

4. Rinse once and wash twice in MABT for 15 min each with rocking.

5. Wash in NTM (which is NTMT without Tween 20) for 2 min.

6. Incubate in NTM containing 4.5 μ l/ml NBT stock and 3.5 μ l/ml BCIP stock per milliliter of solution, for 10 min. Keep the membrane in the dark during the color development reaction. Do not rock.

7. Rinse in H₂O.

A low signal on the spot test may mean either a low total yield of RNA probe or, less commonly, a normal yield of RNA but low incorporation of digoxigenin. In either case, the probe is unlikely to perform well for *in situ* hybridization and should be remade. Once synthesized, probes can be stored in hybridization buffer at -20°C for extended periods.

Day 1: Pretreatments and Hybridization

1. Bleach the embryos in a solution containing 30% H₂O₂: methanol 1:5 for 2 h. Rinse three times in methanol.

2. Rehydrate embryos through 75, 50, 25% methanol/PBT, spending 20 min in each methanol concentration. Wash three times in PBT for 20 min each.

3. Transfer the embryos to 2-ml tubes using a cut pipet tip. To allow efficient washing, the maximum number of embryos processed in a single 2-ml tube during procedure depends on the stage:

Day 7 = 10–15 embryos

Day 8–9 = 7–10 embryos

Day 10 = 2 embryos

4. Treat with 10 $\mu\text{g}/\text{ml}$ proteinase K in PBT at 37°C. One again, the treatment should be adjusted slightly to take the embryonic stage into account. Suggested time of incubation:

Day 7 = 7 min

Day 8–9 = 8 min

Day 10 = 10 min

5. Remove the proteinase K solution and rinse briefly with PBT. Pipet the solution gently onto the embryos, as they are fragile at this point. Postfix for 20 min in 4% paraformaldehyde, 0.1% glutaraldehyde in PBT made fresh from stocks.

6. Rinse once and wash four times with PBT.

7. Rinse with 2 ml hybridization buffer (Table 1) made fresh from stocks. Allow the embryos to settle in the tube.

8. Replace with 2 ml hybridization buffer and incubate at 65°C, three times, for 1 h each.

9. Add 2 ml of prewarmed hybridization buffer and approximately 1 $\mu\text{g}/\text{ml}$ of digoxigenin-labeled RNA probe.

10. Incubate at 65°C overnight.

Day 2: Posthybridization Washes

1. Remove the probe solution, which may be stored for reuse if desired. Rinse twice with prewarmed hybridization buffer at 65°C.

2. Wash for 30 min with prewarmed hybridization buffer at 65°C.

3. Rinse twice with prewarmed washing solution

1 (50% formamide/1 \times SSC/ 0.1% Tween 20 made fresh from stocks) at 65°C.

4. Wash for 30 min with prewarmed washing solution 1 at 65°C.

5. Wash for 1 h with prewarmed washing solution 1 at 65°C.

6. Wash for 30 min with 1:1 washing solution 1/MABT at room temperature.

7. Rinse three times with MABT and then wash four times for 30 min each with MABT.

8. Replace with MABT + 2% BBR (Boehringer Blocking Reagent, Catalog No. 1-096-176) made fresh. Wash for 1 h at room temperature.

9. Block in 2 ml of MABT + 2% BBR + 20% heat-inactivated normal goat serum for 1–2 h.

10. Replace with MABT + 2% BBR + 20% heat-inactivated goat serum containing anti-digoxigenin-alkaline phosphatase antibody (Boehringer Mannheim, catalog No. 1-093-274). For embryos at Day 7 to Day 9 use a 1/2000 dilution of the antibody. For embryos at Day 10 use a 1/5000 dilution of antibody. In both cases, incubate overnight at 4°C.

Day 3: Postantibody Washes and Histochemistry

1. Rinse three times with MABT.

2. Wash four times with MABT for 15 min each.

3. Wash four times with MABT for 30 min each.

4. Wash twice with MABT for 1 h each.

5. Wash twice with NTMT (Table 2) made fresh from stocks for 10 min each.

6. Incubate with NTMT containing 4.5 $\mu\text{l}/\text{ml}$ NBT stock and 3.5 $\mu\text{l}/\text{ml}$ BCIP stock per milliliter of solution. Keep the samples in the dark during the color reaction incubation. Rock for the first 20 min only.

7. Use a stereomicroscope to monitor the progress of the color reaction. When the color has developed to the desired extent (30 min to 3 days), wash three times with PBT. Wash in 50% glycerol/ 0.1% sodium

TABLE 1
Hybridization Buffer

Stock concentration	Final concentration	Volume
Formamide, 100%	50%	25 ml
20 \times SSC, pH 5, with citric acid ^a	1.3 \times	3.25 ml
EDTA, 0.5 M, pH 8 ^a	5 mM	0.5 ml
Yeast ribonucleic acid core particle, 20 mg/ml ^b	50 $\mu\text{g}/\text{ml}$	125 μl
Tween 20, 10% ^a	0.2%	1 ml
CHAPS, 10% ^b	0.5%	2.5 ml
Heparin, 50 mg/ml ^b	100 $\mu\text{g}/\text{ml}$	100 μl
H ₂ O ^a		17.5 ml
Total		50 ml

^a DEPC-treated and autoclaved.

^b Made with DEPC-treated H₂O.

TABLE 2
NTMT

Stock concentration	Volume
NaCl, 5 M	1.0 ml
Tris-HCl, 2 M, pH 9.5	2.5 ml
MgCl ₂ , 2 M	1.25 ml
Tween 20, 10%	5.0 ml
H ₂ O	40.25 ml
Total	50 ml

azide in PBT overnight, then in 80% glycerol/ 0.1% sodium azide in PBT overnight.

CONCLUDING REMARKS

Helpful Hints for Troubleshooting High Background and Low Signal

Proteinase K Conditions

One potential source of high background and low signal is insufficient digestion with proteinase K. Failure to adequately treat the samples will limit permeability of the embryos and will prevent the probe from efficiently entering the embryonic tissues. We find it useful to monitor the progress of the proteinase K digestion by eye. When embryos are being digested, look for small pieces of yolk sac that break free from the embryos. This signifies that digestion is complete. On the other hand, we find that embryos which have not been adequately digested do not become transparent in the hybridization buffer. The proteinase K digestion step is necessarily a compromise. Increasing the length of proteinase K treatment should allow better penetration of the probe. On the other hand, increasing the proteinase K digestion time too much will also cause low signal, since the mRNA in the tissue will be free to diffuse and will be washed out. In practice, overdigested embryos can be identified by tissue that is ragged and broken, especially along the neural tube.

Probe Hybridization/Washing Conditions

Sometimes high background may be eliminated by lowering the amount of probe added to the hybridization buffer. This presumably will result in a reduction in the levels of nonspecific binding. In addition, an extra 1-h wash in washing solution 1 may help to reduce background staining. These conditions are frequently required for Day 10 embryos which are prone to higher background. We find that some specific probes reproducibly generate high background in embryos of all stages. An additional way to lower the background for these probes is to use an RNase treatment, which digests only unhybridized RNA. These optional steps can be inserted between steps 4 and 5 on Day 2. Rinse two times in RNase buffer (0.5 M NaCl, 10 mM Pipes, pH 7.2, 0.1% Tween 20).

Wash for 1 h at 37°C in RNase buffer + 100 $\mu\text{g/ml}$ RNase A + 100 units of RNase T1. Wash three times in RNase buffer. Wash twice with washing solution 1. Be very careful when carrying out these steps because RNase contamination of any solutions used either before or during the hybridization step could lead to reduction or elimination of signal.

Antibody Hybridization/Washing Conditions

High background may also be alleviated by lowering the concentration of anti-digoxigenin-AP antibody. In addition, after antibody washes, leave the embryos in MABT overnight at 4°C without rocking, then rinse and wash again twice for 1 h each before developing the color reaction. These conditions are frequently required for Day 10 embryos.

In Situ Hybridization to Early Embryos and Embryo Fragments

When processing many different groups of small samples it may be easier to process the tissue in 24-well plates (Falcon, Catalog No. 3047) rather than using 2-ml tubes. It is often helpful to place the plate on a black background to facilitate viewing the embryos and to prevent loss of the samples when changing solutions. Hybridization and washing to remove the unhybridized probe should be carried out in a humidified chamber inside a shaking water bath. All washes and incubations are carried out in a volume of 1 ml rather than 2 ml, except for the hybridization, which can be done in 500 μl without shaking. Incubate in washing solution 1 with gentle shaking, 3 \times 30 min for each wash.

REFERENCES

1. Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J., and Ish-Horowicz, D. (1995) *Nature* 375, 787–790.
2. Conlon, R. A., and Rossant, J. (1992) *Development* 116, 357–368.
3. Conlon, R. A. (1996) *in* Whole Mount *in Situ* Hybridization to Mouse Embryos (Krieg, P. A., Ed.), pp. 371–380, Wiley-Liss, New York.
4. Hogan, B. L. M., Beddington, R., Costantini, F., and Lacy, E. (1994) *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY.
5. Abraham, T. W. (2001) *Methods* 23, 297–302.