

MEI Protocol

Whole-Mount In Situ Hybridization On E18 diaphragm

By Xianping Dong

1. Dissect embryos out in PBS + 2mM EGTA; remove as much of the extra-embryonic membranes as possible.
2. Fix in 10 ml 4% PFA in PBS, 1-2h at room temp or 4⁰C o/n.
3. Wash Twice with PTW (= PBS, 0.1% Tween-20)

Pretreatment and hybridization

1. Treat with 50 μ g/ ml proteinase K (aliquot and store at -80⁰C) for 30min at 37C in PTW.
2. Remove proteinase, rinse briefly (care!) with PTW, and post-fix for 20 minutes in 4% PFA in PBS.
3. Rinse and wash once with PTW.
4. Rinse once with 1:1 PTW/hybridization mix. Let embryos settle.
5. Rinse with 1 ml hybridization mix. Let embryos settle.
6. Replace with 1 ml hybridization mix and incubate horizontally 1h/55⁰C.
7. Denature DIG-labeled RNA probe at 80-95⁰C (add 1 μ l probe to 50 μ l pre-warmed hybridization).
8. Add 1 ml pre-warmed hybridization mix @ ~1 μ g/ml DIG-labeled RNA probe (possibly 0.1 μ g/ml is enough).
9. Incubate horizontally at 55⁰C/overnight. Mix after 20-30 min.

- Steps 1-9 are carried out on a roller, and wash for 5 min; 4% fixative should be fresh.

Post-Hybridization washes

1. Rinse twice with prewarmed (55⁰C) hybridization mix.
2. Wash 15 min with 55⁰C with prewarmed hyb mix.
3. Wash 5 min at 55⁰C with prewarmed 1:1 prewarmed hyb mix / TBST [TBST: 150mM NaCl, 10mM KCl, 50mM Tris pH 7.5, 0.1% Tween-20; can do stock 10x].
4. Rinse 3 times with r TBST.

5. Wash 2x30 min with TBST.
6. Replace with TBST + 2% BBR [Boehringer Blocking Reagent, BM 1096 176; Roche11096176001], make 10 % stock in MAB] by heating to dissolve, autoclave, aliquot and freeze] + 20 % heat treated goat serum (56⁰C for 30 min). Incubate for 1-2 hours at room temperature.
(600 µl PBW or TBST + 200 µl 10% BBR + 200 µl Goat serum)
Rinse once with a solution of TBST +2% BBR + 1% serum. (800 µl PBW or TBST + 200 µl 10% BBR + 10 µl Goat serum).
7. Replace with a solution of TBST +2% BBR + 1% serum, containing a 1/1000-2000 dilution of anti-DIG-AP antibody (BM 1093 274). Incubate overnight at +4⁰C or 4-6 hours at room temperature.
 - Don't let samples cool down during 55C washes;
 - Serum is heat-treated at 55-60⁰C, 30 min and store in quick-frozen aliquots at -20⁰C.

Post- Antibody Washes And Histochemistry

1. Rinse 3 times with TBST.
2. Wash 3-5x1h with 1 ml TBST.
3. Wash 2x 10 min with NTMT.
4. Incubate with 1 ml NTMT + 9 µl/ ml NBT (75 mg/ml in 70 % DMF) +7 µl/ ml BCIP (50 mg/ ml in DMF) at RT. Rock for 20 min (Develops faster at 37⁰C, if necessary).
5. When color has developed to the desired extent (30 min to >2 days, changing staining solution regularly), wash 3x with PTW. Refix in 4% HCHO or PFA in PTW, overnight, followed by PTW washes and storage in PTW/ 0.1% azide, at +4⁰C.

If there is too much “pinkish background, can blench embryos in 6% H₂O₂/PBS for one hour, before refixing.

Solutions

Hybridization mix: can be stored at -20⁰C

Stock	Final conc.	To 50 ml
Formamide (Fluka 47671)	50%	25 ml
SSC (20x pH 5 w citric acid!!)	1.3xSSC	3.25 ml
EDTA (0.5M, pH8)	5mM	0.5ml
Yeast RNA (20mg/ml)	50 µg /ml	125 µl
Tween-20 (10%)	0.2%	1 ml
CHAPS (10%)	0.5%	2.5 ml
Heparin (50mg/ml)	100 µg/ ml	100 µl
H ₂ O		17.5 ml

MAB – 100 mM maleic acid, 150 mM NaCl pH 7.5. Can do a 5x stock solution: dissolve first the maleic acid, titrate with NaOH and then add the remaining components

Blocking reagent - Boehringer Blocking Reagent (BM 1096 176) is made up in maleic acid buffer as 10% stock. Heat to dissolve, autoclave, aliquot and freeze.

NBT (Sigma N 6876)- To the bottle containing 1 gm of powder, add 9.1 ml DimethylFormamide (DMF) and 3.9 ml water. Vortex to dissolve. Store at -20°C .

BCIP (Sigma B 8503)- To the 500 mg bottle, add 9.7 ml DMF. Vortex to dissolve. Store at -20°C .

NTMT: Make from stocks on day of use.

5M NaCl	1 ml
2M TrisHCl pH 9.5	2.5 ml
2M MgCl ₂	1.25 ml
10% Tween-20	5 ml
H ₂ O	40.25 ml
Total	50 ml

Note:

Can use PBW throughout the procedure;

Sometimes, 1% Tween 20 can give better results.