Iruela-Arispe Lab
University of California, Los Angeles

WHOLE MOUNT IN SITU HYBRIDIZATION PROCEDURE FOR CHICK (or MOUSE) EMBRYOS

Shelley Thai

FOR PRELIMB BUD AND LIMB BUD EMBRYOS

A. Pretreatment and hybridization of embryos

1. Remove embryos from eggs and dissect extra-embryonic membrances in PBS on ice and opening any cavities such as the heart and the brain to avoid the trapping of reagents.

2. Fix embryos in 10 mL 4% PFA (made fresh) ON at 4oC.

3. Wash embryos 2x5 min each with PBT (PBS + o.1% Tween) at 4oC

DAY1

4. Dehydration: wash for 5 min each at RT with 30%, 50%, and 80% MeOH in PBT, then 2x wih 100% MeOH

→ NOTE: embryos can be stored in 100% MeOH at -20oC for a long time prior to usuage.

5. Rehydration: take embryos through graded MeOH series in the reverse order and then wash 2x 5 min each with PBT at RT

For Limb Bud embryos:

6. Bleach embryos in 6% H2O2in PBT for 1hr at RT
(Stock is 30%)

7. Wash with PBT at RT 3x 5 min each

8. Treat with proteinase K at 10 ug/mL for 15 min at RT for day 4 embryos and 20 min at 37oC for day 5 and 6 embryos

9. Wash 2x 5 min at RT with freshly made 2mg/ml glycine in PBT

10. Wash twice with PBT 5 min each at RT

For Prelimb embryos:

6. Permeabilize embryos with 3x 30 min washes with RIPA buffer at RT

RIPA buffer: 150 mM NaCl
1% Nonidet P-40
0.5% deoxycholate
0.1% SDS
1 mM EDTA
50 mM Tris-HCl
pH 8.0

For both prelimb bud and limb bud embryos

11. Refix the embryos with fresh 0.2%GDA/4% PFA in PBT for 20 min at RT

12. Wash embryos 4x 5 min each with PBT at RT

13. Wash once with pre-hybridization buffer at RT for 5 min

Prehybridization buffer: 50% formamide
5xSSC pH4.5 (pHed with citric acid)
1% SDS
50 ug/ml total yeast RNA (boil for 5 min)
100ul/10ml ssDNA (boil for 5 min)
50 ug/ml heparin
pH 4.5

14, Incubate embryos with prehybridization buffer at 70oC for 1 hr or more

15. Hybridize embryos in hybridization solution for ON with rocking at 70oC

Hybridization solution: prehybridization solution plus 1ug/ml of DIG-labeled RNA probe
(heat probe at 80oC for 5 min prior to usuage)

DAY 2: Washes(It is good to pre-warm all solutions to the respective temperature before use.

16. Wash 3x 30 min each at 70oC with solution #1

solution #1: 50% formamide
5xSSC pH4.5
1% SDS
pH4.5

17. Wash 3x30 min each at 65oC with solution #3

Solution #3: 50% formamide
2xSSC pH 4.5
pH 4.5

18. Wash 3x5 min each with Tris-buffered saline (TBS, plus 2 mM levamisole) containing 0.1% Tween-20.

10XTBST: 1.4M NaCl
27 mM KCl
0.25 M Tris-HCl, pH7.5
1% Tween-20
in ddH20 or DEPC water autoclave

OR 10XTBST: for 100 ml
8g NaCl
0.2g KCl
25mL 1 M Tris pH7.5
10mL 10% Tween-20
QS to 100mL Autoclave

18. Preblock embryos in TBS plus 0.1% Tween-20 plus 10% heat-inactivated sheep serum for 2.5 hr at RT
sheep serum is heat-inactivated at 70oC for 30 min before use

19. During this step, preabsorb the antibody as follows:
a) weigh out 3 mg embryo powder into a microtube
b) add 0.5 mL TBST and heat at 70oC for 30 min. Vortex to help mix.
c) cool on ice and add 5 ul sheep serum and 1 ul anti-DIG-AP conjugated antibody
d) shake gently at 4oC for 1 hr, then spin in a microcentrifuge at 4oC for 10 min.
e) recover supernatant and dilute it to 2 ml with 1% sheep serum in TBST

20. Discard preblocking solution and add 1 mL pre-blocked antibody to embryos for 1-2 min.

21. Replace with fresh preblocked antibody and rock at 4oC ON

DAY3: Washes

22. Wash embryos 3x 5 min each time at RT with TBS plus 0.1% Tween-20.

23. Wash 5x for 1.5 hr each time at RT with TBS plus 0.1% tween-20 at RT

24. Wash ON with TBS plus 0.1% Tween 20 at 4oC.

DAY 4: Histochemistry

25. Wash 3x 10 min each with NTMT (This is usually made fresh from stock because the pH value can change due to the absorption of CO2.)

NTMT: 100 mM NaCl
100 mM Tris-HCl, pH 9.5
50 mM MgCl
0.1% Tween-20
2 mM levamisole
in ddH2O or DEPC-H2)

26. Incubate embryos with detection solution:

Detection solution:

NTMT with 0.25 mg/ml nitroblue tetrazolium (NBT)and 0.13 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate toluidinium (BCIP)
Pre-limb bud are incubated for 5-15 hr
limb bud stages embryos for 1-5 hr
Rock in the dark for the first 20 min only.
Keep in the dark as much as possible.

27. After the detection reaction was deemed complete, embryos were washed twice with NTMT, once with PBT (pH5.5)

28. Embryos can be post fixed with 4% PFA/0.1% GAD in PBT

29. Wash several times with PBT

30. Embryos can then be cleared through a series of 30%, 50%, 70%, and 80% glycerol in PBT.