

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

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FOR PRELIMB BUD AND LIMB BUD EMBRYOS

A. Pretreatment and hybridization of embryos

1. Remove embryos from eggs and dissect extra-embryonic membranes 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 + 0.1% Tween) at 4oC

DAY1

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

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

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% H₂O₂ in 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 usage)

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 ddH2O 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 70°C 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 70°C for 30 min. Vortex to help mix.
 - c. cool on ice and add 5 µl sheep serum and 1 µl anti-DIG-AP conjugated antibody
 - d. shake gently at 4°C for 1 hr, then spin in a microcentrifuge at 4°C 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 4°C 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 4°C.

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 (pH 5.5)
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.