

# RNA Wholemount *In situ* Hybridization

## Day 1:

### Pretreatment and hybridization of embryos (or explants)

**Wear gloves, use only DEPC treated solutions (RNase free!), have a heat block @ 70°**

1. Take embryos from -20°C Methanol (100%) and rehydrate through PBT series:

75% MeOH / 25% PBT 5MIN or 10MIN\*

50% MeOH / 50% PBT 5MIN or 10MIN\*

25% MeOH / 75% PBT 5MIN or 10MIN\*

100% PBT (2X) 5MIN or 10MIN each\*

This and all subsequent washes (Days 1-3) done at room temperature gently rocking unless otherwise stated. Adjust volumes and wash times appropriately:

\*E9.5 and below ~4ml in small snap cap 5MIN each wash (this includes explants)\*

\*E10.0 and above ~10ml in large snap cap 10MIN each wash (to fully equilibrate)\*

2. Bleach with 6% Hydrogen Peroxide in PBT 1HR at RT

Stock H<sub>2</sub>O<sub>2</sub> is 30% stored @ 4° (1ml 30% + 4ml PBT)

3. Wash 3X with PBT for 5MIN each at RT (Increase wash time for larger/older embryos)

4. Treat with (10µg/ml) Proteinase K in PBT for 15MIN at RT

Stock is 10mg/ml stored @ -20° (5µl 10mg/ml + 5ml PBT)

Do not increase time for older embryos, this step is an exception to the rule

Change to a new pipet dropper after this step (avoid Proteinase K contamination)

5. Wash with freshly made (2mg/ml) Glycine in PBT for 5MIN at RT (10MIN for >E9.5 embryos)

6. Wash 2X with PBT 5MIN each at RT (This & all subsequent washes should be adjusted properly)

7. Refix with .2% Glutaraldehyde/4% Paraformaldehyde in PBT for 20MIN at RT

Stock Glutaraldehyde is 8% stored at -20° (125µl 8% Glu + 4.875ml 4% Para)

8. Wash 2X with PBT for 5MIN each at RT

9. Add Prehybridization Solution (~0.25-0.5ml), mix briefly, remove, add final volume

(~1-2ml) of Prehybridization solution. Put at 70° for 1HR. Adjust volume for embryos

From this point on, **maintain 70°** to avoid nonspecific hybridization of probe

10. Add 70° Hybridization Solution(~0.25-0.5ml), mix briefly, remove, add final volume of Hybridization solution (~0.5-1ml in small snap cap tube, 1-2ml for large). Hybridize in smallest volume possible with digoxigenin probe @ [1µg/ml]. Place mineral oil over surface of solution (prevents evaporation), then place at 70° overnight in heat blocks. Again, in appropriate volumes

### **Solutions:**

**PBT:** 1X PBS (DEPC H<sub>2</sub>O) with .1% Tween-20

**50 ml of 4% Paraformaldehyde (PFA):** Dissolve 2g of PFA in ~25ml DEPC H<sub>2</sub>O with 6µl 10N NaOH, place at 65° for ~1HR, chill to @ least RT, then add DEPC H<sub>2</sub>O to 45ml and add 5ml of 10x PBS

-Aliquot to ~10ml volumes and store @ -20° (good for ~a month)

**Prehyb and Hybridization Solutions:** 50% Formamide, 5X SSC pH 4.5 (Use citric acid to pH), 50µg/ml yeast RNA, 1% SDS, 50µg/ml Heparin

-Aliquot to ~10ml and store @ -20° (good for a long time)

## Day 2:

performed on rotator unless otherwise stated, increase volume & wash time for >E10 (see Day 1)

1. Transfer embryos with as little volume possible and no oil to a fresh snap cap tube
2. Wash 2X with **prewarmed 70°** Solution I using ~4ml for **30MIN each at 70°**
3. Wash 1X with 1:1 mix of Solution I:Solution II, **prewarmed to 70° for 10MIN @ 70°**
4. Wash 3X with Solution II for **5MIN each at RT** (as y'day: adjust wash time for full equilibration)
5. Wash 2X with 100µl/ml RNase A in Solution II for **30MIN each at 37° H<sub>2</sub>O bath**  
Put on **rocker at RT for 5MIN initially** in first wash to help RNase equilibrate more rapidly into embryo. Stock RNase A is 10mg/ml (40µl + 3.96ml Solution II)
6. Wash 1X with Solution II for **5MIN at RT**
7. Wash 1X with Solution III for **10MIN at RT**
8. Wash 2X with Solution III for **30MIN each at 65°**
9. Wash 3X with TBST with **2mM Levamisole** for **5MIN each at RT**  
Levamisole inhibits mammalian alkaline phosphatase (AP) activity (24mg/50ml)
10. Preblock embryos with 10% sheep serum in TBST (with **Levamisole**) for **2.5HR @ RT in eppendorf tube**. Block endogenous AP in sheep serum: heat @ 70° for 30MIN, store @ -20° or 4°)
11. While blocking embryos, weigh out 3mg of Embryo Powder (stored @ 4°) add 0.5ml of TBST (with **Levamisole**) in eppendorf tube & place at **70° for 30MIN**, **vortex** intermittently/often throughout & at least 1MIN at the end. This is to "expose" as many sites as possible on embryonic mouse proteins & block nonspecific Ab binding. (This volume is per 1 embryo)
12. **Cool** on ice, add sheep serum to 1% and goat or sheep anti-dig-AP conjugated Ab @ 1µl/0.5ml  
Ab can be inactivated/denatured at high temperatures, be sure to cool the solution
13. Rotate the Ab-blocking solution and Ab for **1HR at 4°**
14. Spin in microfuge for **10MIN at 4°**
15. Dilute **supernatant** to 2ml, add ~1.5ml of 1% sheep serum in TBST (with Levamisole)
16. Remove Blocking Serum, add ~0.5ml of dig-Ab Solution, remove, and add final hybridization volume of ~1.5ml per .5ml of blocking solution (~5X volume of embryos) at **4° O/N on rocker** in eppendorf. Use small snap caps if necessary (>E10 or multiple "older" embryos)

**Solutions:**

20ml Solution I: 10ml Formamide, **4ml 20X SSC pH 4.5**, 1ml 20% SDS, 5ml DEPC H<sub>2</sub>O

40ml Solution II: **4ml 5M NaCl**, 4ml 1M Tris pH 7.5, **40µl Tween-20**, 32ml DEPC H<sub>2</sub>O

20ml Solution III: 10ml Formamide, **2ml 20X SSC pH 4.5**, 8ml dH<sub>2</sub>O

50ml TBST: **1.4ml 5M NaCl**, 135µl 1M KCl, **1.25ml 1M Tris pH 7.5**, 50µl Tween-20, **47.2ml dH<sub>2</sub>O**

### **Day 3:**

#### **Post Ab binding, AP substrate addition**

All washes done on rotator at RT unless otherwise stated, make sure **ALL** solutions are fresh and with **2mM Levamisole**. **NBT** and **BCIP** Solutions are photosensitive, make sure **all** solutions are protected from light. As always, adjust volumes appropriately

1. Transfer (if Ab binding performed in an eppendorf tube) to a fresh small snap cap tube, or large snap cap for older embryos (>E9.5) or high volume of embryos (e.g., multiple #s of E9.5 embryos)
2. Wash 3X with TBST with Levamisole for **10MIN each** at RT
3. Wash 5X with TBST with Levamisole for **1HR each** at RT
4. Wash 3X with NTMT with Levamisole for **10MIN each** at RT
5. Transfer with as little volume as possible to eppendorf tube, or use a small snap cap if this volume is insufficient
- 6.. Add 0.5ml of freshly made NBT/BCIP Solution\* to embryos, remove, add final substrate volume ~1-1.5ml (larger appropriate volume if not in eppendorf tube) in **light protected tube**, place on rocker at RT for **20MIN**  
\*NBT added at 4.4µl/ml, BCIP added at 3.3µl/ml
7. Remove from rocker and place stationary until sufficient signal is obtained  
Continue to keep embryos and solution in the dark  
If not removed from rocker substrate will precipitate out of solution  
Signal can appear anywhere from **30MIN to overnight**
8. When reaction is complete:
  - A) Wash 2X in NTMT for **10MIN** at RT while in dark
  - B) Wash in PBT **pH 5.5** for **10MIN** at RT  
Both the acidity and phosphates inhibit AP activity
9. Alternately, to completely clear embryos, dehydrate in MeOH/PBT series 25%, 50%, 75%, 100% for **20MIN** each at RT on rocker
10. Put in 1:2 Benzyl Alcohol:Benzyol Benzoate Solution for maximum of **2HR** then back through MeOH/PBT series for the following steps:
11. Post-fix embryos in 4% Paraformaldehyde in PBT + 0.1% Glutaraldehyde for **1HR**  
Stock Glutaraldehyde is 8% stored at -20° (125µl 8% Glu + 4.875ml 4% Para)
12. Wash 2X with PBT for **10MIN each**
12. Store in PBT at 4°

#### **Solutions:**

**50 ml TBST:** 1.4ml 5M NaCl, **135µl 1M KCl**, 1.25ml 1M Tris pH 7.5, **50µl Tween-20**, 47.2ml dH<sub>2</sub>O

**50ml NTMT:** **1ml 5M NaCl**, 2.5ml 2M Tris pH 9.5, **250µl 1M MgCl<sub>2</sub>**, 50µl Tween-20, **46.2ml dH<sub>2</sub>O**

**NBT:** .0075g into 100µl 70% Dimethyl Formamide

**BCIP:** .0050g into 100µl 100 Dimethyl Formamide