

# Frog *in situ* Hybridization Protocol

## Part 1: Probe Preparation

### 1. Linearize Plasmid (Restriction Digest)

Xul (1ug) of plasmid DNA  
1ul NEB restriction enzyme  
2ul 10X NEB buffer  
2ul BSA (if required)  
Xul dH2O (bring up to 20ul)

- put in appropriate water bath (usually 37C) for 2-5hrs
- scale up for larger amounts of DNA

### 2. Clean up linear DNA

#### **Option 1: phenol:chloroform extract and NaOAc ppt**

- bring up volume of DNA solution to 100ul with dH2O
- add 100ul of phenol: chloroform solution (do this in fume hood)
- vortex for 10s
- centrifuge for 2 min at max speed
- keep supernatant and put into a new tube
- add: 1ul glycogen  
10ul NaOAc  
200ul cold 100% ethanol
- put into freezer overnight

- centrifuge 20min at max speed in cold room
- discard supernatant
- add 1ml of 75% ethanol
- centrifuge 20min at max speed in cold room
- discard supernatant and let pellet dry
- add 30ul dH2O

#### **Option 2: PCR Qiagen Kit Cleanup**

Follow manufacturers instructions

### 3. Determine concentration with nanospec.

### 4. Transcription Reaction

- Xul linear template (1ug)
  - 4 ul of 5X transcription buffer (or 2ul of 10X transcription buffer)
  - 2ul DIG RNA labeling mix
  - 2ul of the appropriate polymerase (T7, T3, or SP6)
  - Xul dH<sub>2</sub>O
- put in 37C water bath for 2-3hrs

### **5. Lithium Chloride PPT**

- bring volume up to 50ul
  - add 25ul Lithium chloride solution from Ambion
  - incubate overnight in freezer (-20C)
  
  - centrifuge 20min max speed
  - discard supernatant
  - add 1ml 70% ethanol
  - centrifuge 20min max speed
  - discard supernatant and let pellet dry
  - resuspend in 30ul dH<sub>2</sub>O
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### **Optional Steps to precede LiCl ppt**

#### ***Remove DNA template (Dnase trt.)***

*add 1ul DnaseI, mix gently*

- put in 37C water bath for 30 min

#### ***Put through RNA mini spin column (remove unincorporated nucleotides)***

- snap off top then bottom
- centrifuge at 3500 for 1min
- discard buffer and blot bottom on clean Kim wipe
- add sample to middle of column
- centrifuge for 4min at 3500

### **Optional Step after LiCl ppt**

#### ***For large probes it may be useful to hydrolyze:***

- Add 50  $\mu$ l Sodium Bicarb/Sodium Carb solution
- Heat for 35-60 minutes at 60oC
- Add:
  - 200  $\mu$ l H<sub>2</sub>O
  - 25  $\mu$ l of 3M NaOAc
  - 600  $\mu$ l EtOH

*-Spin for 10 min and remove supernatant*

## **7. Check RNA probe on a Formaldehyde Gel**

### **a) Make the gel:**

agarose	0.5g
10X MOPS buffer	5ml
dH2O	43.5ml

- heat this in microwave 1-2mins
- measure volume of melted solution in graduated cylinder
- top up to 48.5ml with dH2O
- add 2.55ml new (less than 6 months old) 37% formaldehyde
- pour into mold and wait at least 30 min (do this in fume hood)

### **b) Prepare samples**

-add

- 7.5ul of RNA solution (this should be around 1ug of RNA-dilute or scale up if not)
- 7.5ul of RNA loading buffer (premixed with EtBr and BPB, Sigma R-4268)

-heat at 65C for 10 min and then put on ice for 5min

### **c) Prepare ladder**

- remove aliquot from -80C freezer (each aliquot is 1ug in 3ul)
- dilute up to 7.5ul
- add 7.5ul RNA loading buffer  
(depending on the ladder you use you may have to heat at 65C for 10 min)

### **d) Run Gel**

- put gel into box and add 1X MOPS buffer to fill line
- load samples (15ul of each)
- set voltage at 65-70V (no higher)

e) View gel as soon as the purple reaches just below halfway mark

## Part B: Embryo Preparation, Hybridization and Staining

This protocol is a modification of (see: Early development of *Xenopus laevis*: a laboratory manual. Sive HL, Grainger R, Harlard R. [Cold Spring Harbor Laboratory Press](#), 2000.).

### Day 1

1. dechorionate embryos in 0.1X MBS plus few drops of 1% tricaine
2. Fix in 4% PFA overnight 4C (can be reduced to 5hrs at room temp.)  
(make 4% PFA from 16% PFA stock and PBS-)

### Day 2

1. Rinse in PBS 3X 15min (*do in netwells and black baskets*)
2. Cut off the heads of the embryos-exposing the archenteron/foregut cavity
3. Dehydrate in methanol series. (***methanol rather than ethanol prevents bubbles in H<sub>2</sub>O<sub>2</sub> step***)  
25% to 50% to 70% to 80% to 90% to 100% to 100%  
10 min in each (use net wells and black baskets)  
*After final change put in freezer overnight in a sandwich bag to prevent evaporation.*

### Day 3

1. Rehydrate in methanol series (reverse the above Day 2, step 3 and end in PBS)
2. Wash in PBT (PBS + 0.1% tween-20)  
3X 15min (use netwells in black baskets)

#### **Optional**

- at this point some probes benefit by treating with Proteinase K*
  - however I don't use unless I get a weak signal*
  - Proteinase K ~5-10 min. (100µl of 10mg/ml PK/100ml PBT)
  - Wash 3 X 5 min
3. Rinse in 0.1M triethanolamine  
2X 5min (*use net wells in clear culture dishes*)
  4. Add 12.5ul acetic anhydride to 4ml of triethanolamine-swirl, incubate 5min  
(*use acetic anhydride at full strength and always make just before use*)
  5. repeat step 4
  6. Wash 3X 15min in PBT  
*take embryos out of net wells and put directly into culture dishes*
  7. Bleach embryos on light box:  
2ml H<sub>2</sub>O<sub>2</sub> (30% soln)  
2ml formamide

36ml 2X SSC

*-this takes 1-2hrs or until embryos are white*

8. Wash in 2XSSC 1X 15min (*use netwells and black baskets*)

9. Wash in PBT 2X 15min (*use netwells and black baskets*)

10. Transfer to 1.5ml tubes and add hybridization buffer (without the probe).

Incubate overnight at 60C

(or put at 4C for weekend then 60C overnight on Monday)

*-keep hybridization buffer in freezer (-20C)*

*Put approx. 10mls of hybridization buffer in 60C incubator for probe the next 2 days*

#### **Day 4**

1. Add probe to warmed hybridization buffer at appropriate conc. (usually 1ug/ml for most probes, but has to be determined each time). The quality and quantity of the probe is the most important aspect of good staining.

2. Add probe/hybridization buffer solution to embryos, approx 250-500ul per 8 embryo heads. Incubate at 60C overnight.

*Put 2XSSC and 0.2XSSC in 60C for next day*

#### **Day 5**

1. Remove probe and save in labeled tubes (if staining does not work run a sample of this on a gel to see if RNA is degraded)

2. Add warm hybridization buffer and incubate for 30min at 60C

*Do the next steps in netwells and clear plastic dishes*

3. Wash with warm 2X SSC 4X 30min at 60C

4. Wash with warm 0.2X SSC 4X 30min at 60C

5. Wash with room temp. 0.2XSSC 1X 15min

6. Wash in MAB 3X 15min

7. Block for 2-3hrs in Block Soln.

1 BMB stock: 1 lamb serum: 8 MAB

*put extra in freezer for next time*

*-put embryos back into tubes*

8. Add 1ml of antibody solution to each tube

.63 ul anti-DIG-AP

1.0 ml Block Soln

incubate overnight or weekend at 4C in tubes on shaker

#### **Day 6**

*Put embryos in net wells and black baskets*

1. wash with MAB 6X 1hr

*Put net wells into clear culture dishes*

2. wash in AP buffer 2X 30 min

*take embryos out of net wells and put directly into clear culture dishes*

**3.** Incubate in NBT/BCIP 36ul/28ul 10mls AP buffer + 2% PVA (heat to get PVA into solution)

-keep in the dark and check staining often under microscope

**4.** Rinse in AP buffer (no PVA) 3X 10 min

*If there is alot of background to remove or if you want the color to look blue then do following steps:*

**5.** Put embryos directly into 100% Methanol to clear background and turn stain blue rather than purple. Watch this-you can fade your staining tooo much!!! and rehydrate quickly (2min each through Methanol series ending in PBS). Finally fix in 4% PFA and take pictures within 24hrs-make sure you plan the time for this.

Alternatively, put embryos from 100% Meth to BB:BA solution to clear, photograph immediately.

If you want to section the embryo, you need to overstain, then fix in 4% PFA overnight and wash in PBS and then put directly into infiltration solution.