

## **In Situ Hybridization: digoxigenin labeling of frozen sections**

### **Day1- In Situ Hybridization on Frozen Sections**

1. Cryoembed eyes or heads as detailed in 'Cryoembedding protocol'. Cryosection 10-20µm thick sections and store at -70°C until used for *in situ* hybridization. \*\* note- the length of fixation can drastically impact your results. A typical chick eye should be fixed for approximately 3 hours (or longer) at room temperature.
2. Take out slides and let air dry for several minutes, then encircle the tissues with a hydrophobic pap pen. Let sit at room temperature for another twenty minutes (or more) to ensure that the tissue doesn't loosen during the subsequent hybridization steps.
3. Rinse in 1X PBS containing active DEPC. Use in hood and dispose of DEPC in container in hood to be autoclaved and disposed.
4. Optional- (but strongly recommended!) - Treat sections with 10µg/ml proteinase K in PBS at room temp for 1 min. (Prot. K is stored in freezer door in 10 mg/ml aliquots, so dilute 1:1000 for working concentration).
5. Rinse sections with 1X PBS

### **Acetic Anhydride Treatment (Optional-but necessary for cell culture dishes)**

- 1) Add acetic anhydride to a final concentration of 0.25% to 100mM triethanolamine, pH8.0.
  - 2) Incubate for 10 min
  - 3) Rinse in 1X PBS
6. Prehybridize sections with Hybridization buffer for at least two hours at 65°C. (Make sure humidity chamber has plenty of water in it otherwise tissue sections will dry out. If there is not a good seal on the chamber add parafilm around the edges to keep it sealed).
  7. Add 75-125µl of 400ng/ml (range is 100ng-1000ng/ml) of probe diluted in Hyb solution to each slide
  8. Cover sections with coverslip (use only 50 or 60mm coverslips to prevent tissue drying!), being careful not to get too many bubbles under coverslip.
  9. Hybridize at 55-65°C overnight (go with 65°C first and lower if too little signal is detected).

### **Day2 - Post Hybridization Processing**

\*\* set water baths to 42° and 60°C prior to beginning any washes!!

1. Remove coverslips by soaking slides in 2X SSC at 37°C for a few minutes in a coplin jar.
2. Incubate slides in 50% formamide in 2X SSC at 60°C, 2X for 30 min ea.
3. Rinse slides in 37°C 2X SSC three times
4. Optional (but recommended) -Treat with 50 ml RNase A (located in - 20°C) and RNase T1 (located in 4°C) in 2X SSC at 37°C for 15 min (As per Nieto, et al (1996), RNase treatment can decrease the specific signal – if you are looking for a message that is not abundant, and are having trouble detecting a signal, then try decreasing the amount of RNase or the delete RNase step altogether)

For 50 ml:

50µl of 10mg/ml stock RNase A (100-200µl via old protocol; 12.5µl for Valeria)

25 µl of 100,000 U/ml stock RNase T1 (50-100µl via old protocol; 5µl for Valeria)

-> this yields a 10µg/ml final concentration of RNase A, and 50 U/ml of RNase T1.

5. Wash in 50% formamide in 2XSSC with 0.1% CHAPS at 60°C for 15 min
6. Rinse sections in 0.2X SSC with 0.1% Tween 20 for 10 min at 42°C
7. Decant 1/2 of 0.2X SSC/0.1%Tween20 and add Buffer 1
8. Decant all of above solution and add Buffer 1
9. Block sections with 2% Roche Blocking Buffer (diluted in maleate buffer) for 1 hr at room temp.
10. Decant blocking buffer and remove all excess with kimwipe so as not to dilute antibody
11. Incubate sections with anti-Dig antibody (Roche) at 1:3000 (range 1:1000-1:3000) diluted in 2% blocking buffer for 2hrs at room temp
12. Wash in [Buffer 1](#) 3X, 15 each at room temp
13. Rinse in [Buffer 3](#)
14. Develop in NBT/BCIP (35µl/35µl) in 10 ml's Buffer 3 overnight at room temperature (previous protocols called for 37°C overnight but this is too fast for many probes).
15. Rinse in TE and coverslip with aquamount (do not use xylene based mounting media and do not wash in alcohols since this will wash the precipitate away).

\*\* some tissues (older and bony) contain endogenous alkaline phosphatase activity and require quenching with a specific blocker (don't know name off hand)

ORDERING INFO:

Catalog #	Description	Company	Size	Contact	CORE
<b>Probe synthesis / pre-hybridization</b>					
10777019	RNAse Out	Invitrogen	5000U	CORE	Y
1277073	RNA labeling mix	Roche	20rxn		Y
	Transcription Buffer	Ambion			N
D-2532	Denhardt's Solution (50X)	Sigma		1.800.325.3010	
776785	DNase I (RNAse free)	Roche	10,000U	CORE	Y
25530-015	Proteinase K (fungal)	Invitrogen	100mg	CORE	Y
18018010	SP6 RNA Polymerase	Invitrogen	500U	CORE	Y
18033019	T7 RNA Polymerase	Invitrogen	2500U	CORE	Y
<b>Post-hybridization</b>					
109 142	RNase A (from bovine pancreas)	Roche	25mg	1.800.428.5433	Y
109 193	RNase T1 (from Apeargillus)	Roche	100,000 U/ml	1.800.428.5433	Y
	575031LFormamide	Sigma	1L		Y
15515026	Formamide (Ultrapure)	Invitrogen	442.5ml	CORE	Y
	CHAPS				
	Tween-20				
	20X SSC	Invitrogen			
1 093 274	Anti-Digoxigenin-AP, Fab frag.	Roche	150 U (200µl)	1.800.428.5433	
1 383 213	NBT solution	Roche	3ml (300mg)	1.800.428.5433	
1 383 213	BCIP solution (50mg/ml)	Roche	3ml (150mg)	1.800.428.5433	
1 096 176	Blocking Reagent	Roche	50g	1.800.428.5433	