

## DIG-LABEL IN SITU HYBRIDIZATION

### Day 1

1. Section embryos using superfrost slides (fresh frozen or over night fixed tissue is best).
2. Air dry
3. Fix in 4% PFA/PBS for 10min at RT
4. Wash 3 times for 3min in PBS
5. Treat section with Prot K solution for 5 min at RT
6. Repeat step 3
7. wash 3 times for 3 min with PBS
8. Acetylate for 10 min at RT  
Acetylation buffer for 400 ml contains 395 ml H<sub>2</sub>O, 5 ml Trietanolamine 0.7 ml conc HCL well mixed. As you are dipping the slides in acetylation buffer add 1 ml anhydride, mix by dipping the slides several times and acetylate with constant stirring on a magnetic stirrer.
9. Wash 3X for 5 min in PBS.
10. Place 700 µl hybridization buffer on each slide. Incubate at RT for 2-16hrs in a 5XSSC humidified chamber.

### HYBRIDIZATION (DAY 1 OR 2)

11. Put 1 µl probe DIG RNA (0.1 µg/µl) in 100µl hybridization solution. Heat at 80°C for 5min and put immediately on ice.
12. Pour off hyb buffer from slide, dab off the edge with kimwipe to remove excess hyb buffer and put 100µl of probe in hyb buffer per slide.
13. Coverslip the slides and place them in black incubation boxes (separate box for each probe) humidified with 5XSSC, 50% Formamide.
14. Place black boxes in 72°C oven overnight.

### WASHES AND STAINING (DAY 2 OR 3)

15. Rack the slides and place rack in a container with 5XSSC at 72°C to remove coverslips.

15. Transfer uncoverslipped slides to a new rack which is in a container of 0.2XSSC at 72°C and wash (let it sit) for 1 hr.
16. Transfer rack to 0.2XSSC at RT for 5 min.
17. Transfer rack to buffer B1 at RT for 5 min.
18. Incubate slides with B1 containing 10%HINGES for 1 hr at RT (either in the rack or on the horizontal slide).
19. Remove block from step 19 and add 0.5ml anti DIG ab (1/3000 in B1 with 1%HINGES) to each slide.
20. Place in humidified chamber O/N at 4°C.

#### DAY 3 OR DAY 4

21. Rinse 3 times for 5 min with buffer B1
22. Equilibrate with Buffer B3 for 15 min
23. Add 1 ml B4 buffer
24. Incubate at RT in a humidified chamber in the dark until color develops, usually at least overnight.
25. Stop reaction by washing with TBST at RT 4X for 5 min each.
26. Pour off washing solution.
27. Preheat slides to 60°C on a heating block (this will dry the slides).
28. Coverslip with DAKO Cytomation Glycergel Mounting Medium (C0563) at 60°C
29. Indulge in results.

#### MAKING DIG LABELED PROBE

1. Linearize 10ug of plasmid (T7, or SP6 promotor)
2. Purify with gel purification columns or Extract with Phenol /Chlorform /Isoamylalcohol and ppt with 2.5 volumes EtOH and 1/10 Vol sodium acetate
3. Resuspend in Rnase free TE
4. For Transcription, follow the protocol provided in the kit (Roche cat # 1-175 025).
5. Remove unincorporated NTPS by passing through G50 column.
6. Resuspend the probe (50µl) in 200µl Hyb buffer to prevent degradation and freeze/ thaw.
7. Store at -20°C.

## SOLUTIONS

### **0.2m Phosphate Buffer (PB) pH7.2 4 L**

165.3 g Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O (MW268.07)

25.6 g NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O (MW 137.99)

Water to 4L

### **4% Paraformaldehyde in PBS (400 ml)**

200ml H<sub>2</sub>O

10μl 10N NaOH

16 g paraformaldehyde powder (PFA)

200 ml 0.2M PB pH7.2

3 g NaCl

Heat water to 70°C on hot/stir plate and add PFA.

Stir with magnetic stir bar to even suspension.

Add 10μl NaOH. When suspension clears add 0.2MPB and 3 g of NaCl.

Filter and chill on ice.

### **PBS (0.1MPB 0.15MNaCl) pH7.2 4L**

2L 0.2M PB pH7.2

35 g NaCl

Water to 4L

### **Proteinase K buffer (ProtK 1ug/ml in 50mM TrisHCl pH7.5 and 5mM EDTA 400 ml)**

5 ml 0.5M EDTA pH8.0

20 ml 1.0M TrisHCl pH7.5

20 μl Prot K @20 mg/ml

H<sub>2</sub>O to 400ul

### **Hybridization Buffer**

50% Formamide (25ml)

5XSSC 12.5 ml of 20X

5X Denhardtts 5ml of 50X

250 ug/ml bakers yeast RNA (Sigma R6750) 1.25 ml of 10mg/ml

100μg/ml salmon sperm DNA (Gibco 10mg/ml) 500μl

To 50ml with H<sub>2</sub>O

**Buffer B1 (TBS/0.1M TrisHCl pH 7.5, 0.15M NaCl) 1L**

100 ml 1M Tris  
30 ml 5M NaCl  
To 1L with H<sub>2</sub>O

**Buffer B3**

0.1M TrisHCl pH9.5  
0.1M NaCl  
50 mM MgCl<sub>2</sub>

**NBT /BCIP for Color Development**

Roche (11681 451 001)

For 10 ml

200µl stock solution  
0.24 mg/ml Levamisole (optional)  
0.1% Tween 20 (100µl of 10%)  
10 ml Buffer B3

Vector (SK5400)

For 5 ml

2 drops of NBT  
2 drops of BCIP  
1 drop Levamisole (optional)  
0.1% Tween 20 final (50µl of 10%)  
5 ml B3