DIG-LABEL IN SITU HYBRIDIZATION

Tissue Preparation: Using fresh frozen tissue will give the maximum signal but histology quality may be sacrificed. An overnight fixation would be optimal for obtaining very good sensitivity while preserving histology. (When testing fixation times and sensitivity, fresh frozen was the most sensitive, overnight fixed tissue was the second most sensitive. Fixation times less than o/n were not as sensitive. However, when combining in situ with immunohistology, shorter fixation times might be necessary to detect protein.

Day1

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

***When using fresh frozen tissue skip steps 5-7.**

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 H2O, 5 ml Trieth anolamine 0.7 ml conc HCL well mixed. As you are dippling the slides in acetylation buffer add 1 ml acetic 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 ul hybridization buffer on each slide. Incubate at RT for 2-16hrs in a 5XSSC humidified chamber.

HYBRIDIZATION (DAY 1 OR 2)

11. Put 1 ul probe DIG RNA (0.1ug/ul) in 100ul hybridization solution. Heat at 80oC 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 100ul of probe in hyb buffer per slide.
13. Coverslip the slides and place them in incubation (slide) boxes (separate box for each probe) humidified with 5XSSC, 50% Formamide.
14. Place black boxes in 65- 72oC oven overnight.

WASHES AND STAINING (DAY 2 OR3)

15. Rack the slides and place rack in a container with 5XSSC at 65-72oC to remove coverslips.
16. Transfer uncoverslipped slides to a new rack which is in a container of 0.2XSSC at 65-72oC and wash (let it sit) for 1hr.
17. Transfer rack to 0.2XSSC at RT for 5 min.
18. Transfer rack to buffer B1 at RT for 5 min.
19. Incubate slides with B1 containing 10%HINGS for 1 hr at RT (either in the rack or on the horizontal slide. If incubating on the horizontal slide add 1-2 ml of goat serum to the container of B1before removing the slides. This will facilitate easy application of the B1 with 10% GS.
20. Remove block from step 19 and add 0.5ml anti DIG ab (1/5000 in B1 with 1%HINGS) to each slide.
21. Place in humidified chamber O/N at 4oC, or for 1-2 hours at RT.

DAY 3 OR DAY 4

22. Rinse 3 times for 5 min with buffer B1
23. Equilibrate with Buffer B3 for 15 min
24. Add I ml B4 buffer. It is best to incubate developing solution either under a cover slip, or you can lower the slide onto the solution on parafilm. This prevents crystal formation due to oxidation.
25. Incubate at RT in a humidified chamber in the dark until color develops, usually at least overnight.
26. Stop reaction by washing with TBST at RT 4X for 5 min each.
27. Pour off washing solution.
28. Preheat slides to 60oC on a heating block (this will dry the slides).

29. Coverslip with DAKO Cytomation Glycergel Mounting Medium (C0563) at 60oC
30. Indulge in results.

MAKING DIG LABELED PROBE

Either:

1. Linearize 10ug of plasmid (T7, T3, or SP6 promoter)
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

Or : Preferred method

1. PCR your gene of interest including a 3' promoter sequence (T7, T3, or SP6) at the 3' end.
2. Gel purify your fragment.
3. Resuspend in Rnase free EB
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 (50ul) in an additional 100ul Hyb buffer to prevent degradation and freeze/ thaw.
7. Store at -20oC.

SOLUTIONS

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

165.3 g Na2HPO4.7H2O (MW268.07)
25.6 g NsH2PO4.H2O (MW 137.99)
Water to 4L

4% Paraformaldehyde in PBS (400 ml)

200ml H2O
10ul 10N NaOH
16 g paraformaldehyde powder (PFA)
200 ml 0.2M PB pH7.2
3 g NaCl

Heat water to 70oC on hot/stir plate and add PFA.
Stir with magnetic stir bar to even suspension.
Add 10ul 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 ul Prot K @20 mg/ml
H2O to 400ul

Hybridization Buffer

50% Formamide	(25ml)
5XSSC	12.5 ml of 20X
5X Denhardts	5ml of 50X
250 ug/ml bakers yeast RNA (Sigma R6750)	1.25 ml of 10mg/ml
100ug.ml salmon sperm DNA (Gibco 10mg/ml)	500ul

To 50ml with H20

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

100 ml 1M Tris
30 ml 5M NaCl
To 1L with H2O

Buffer B3
0.1M TrisHCl pH9.5
0.1M NaCl
50 mM MgCl2

B4
NBT /BCIP for Color Development

Roche (11681 451 001)
For 10 ml

200ul stock solution
0.24 mg/ml Levamisole (optional)
0.1% Tween 20 (100ul 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 (50ul of 10%)
5 ml B3