

DOUBLE IN SITU HYBRIDIZATION PROTOCOL

(Adapted from Marc's in Fishell Lab by Sohyun Ahn June, 2005)

I. Tissue Preparation

1. Fix embryos in 4% PFA at 4°C or perfuse/fix postnatal organs.
2. Wash tissue with cold PBS (1-3 times) and transfer to 30% sucrose/PBS at 4°C until tissue sinks to the bottom.
3. Blot off excess sucrose with Kimwipe and let it sit in OCT for at least 15min before freezing in liquid nitrogen/isopentane.
4. Cut sections at 12-14µm on a cryostat.

II. Pretreatment of Slides (DAY 1)

1. Air dry slides for at least 1hr.
2. Fix in 4% PFA for 10min at 4°C.
3. Wash 2x5min with PBS.
4. Treat with 1.5% H₂O₂ in 100% MeOH for 15min at RT (47.5ml MeOH + 2.5ml 30% H₂O₂).
5. Wash 2x 5min with PBS.
6. Treat with 0.2M HCl in water for 8min at RT.
7. Wash 2x 5min with PBS.
8. Digest in Proteinase K for 5min at RT (10µg/ml in PBS; Roche liquid form).
9. Short wash in PBS.
10. Post-fix in 4% PFA for 10min at 4°C.
11. Wash 1x5min with PBS.
12. Acetylate 10min at RT in freshly prepared 0.1M TEA. (1.85g TEA + 100ml water + 450µl 10N NaOH + 250µl acetic anhydride to pH 7.5)
13. Wash 3x5min with PBS and take off excess PBS.
- (14. Allow slides to air-dry)

III. Hybridization

1. Dilute both DIG and FL probes (50-100ng/ml final. Generally probe is 200ng/µl so dilute first 1:10 in TE, then use anywhere from 0.5µl to 5µl) in hybridization buffer and heat at 80°C for 5min.
2. Add 250µl hot probe to slide and overlay with plastic HybSlip. Hybridize O/N at 55°C.

IV. Post-Hybridization Washes (DAY 2)

1. Pre-warm High Stringency wash, RNase Buffer, and SSC.
2. Float off coverslips by incubating slides in 5xSSC.
3. Place in HIGH STRINGENCY wash (prewarmed) for 30min at 65°C.
4. Wash in RNase buffer 3x10min at 37°C.
5. Treat with RNase A (20µg/ml) for 30min at 37°C.
6. Wash in RNase buffer 15min at 37°C.
7. Wash in High Stringency wash 2x20min at 65°C.
8. Wash in 2xSSC for 15min at 37°C.

9. Wash in 0.1xSSC for 15min at 37°C.
10. Wash with PBT for 15min at RT.
11. Place sections horizontally in a humid box. Block with freshly made 1% BB1 (BM) at 37°C for 30min.
12. Add 250µl of anti-fluorescein POD (1:300, BM, in 1% BB1) and place in a humidified chamber O/N at 4°C.

V. DAY 3

1. Wash with TNT (pH 7.5) 3x 1hr.
2. Block tyramide (thaw 0.5% BB2 from -20°C, warm to 37°C for use. No dilution necessary!). Incubate slides with 0.5% BB2 (Renaissance) for 30min at 37°C.
3. Apply Tyramide-FITC to each slide (TSA, NEN) (1:50 in amplification buffer) for 30min-1hr in the dark at RT.
4. Wash with TNT, pH 7.5 (can check before washing if you use a coverslip.)
5. Check signal under scope and can repeat step 11 to get stronger signal.
6. Wash several times with TNT.
7. Quench POD with 0.3% H₂O₂ in PBS for 15min at RT.
8. (Or heat inactivate the enzyme by incubating at 85°C for 15min)
9. Wash 2x 5min with PBS.
10. Block with freshly made 1% BB1 (BM) at 37°C for 30min.
11. Add 250µl of anti-DIG POD (1:100, BM, in 1% BB1) and place in a humidified chamber O/N at 4°C.

VI. DAY 4

1. Wash with TNT (pH 7.5) 3x 1hr.
2. Block tyramide (thaw 0.5% BB2 from -20°C, warm to 37°C for use. No dilution necessary!). Incubate slides with 0.5% BB2 (Renaissance) for 30min at 37°C.
3. Apply Tyramide-Cy3 to each slide (TSA, NEN) (1:50 in amplification buffer) for 30min-1hr in the dark at RT.
4. Wash with TNT, pH 7.5 (can check before washing if you use a coverslip.)
5. Check signal under scope and can repeat step 3 to get stronger signal.
6. Wash several times with TNT or PBS.
7. Counter-stain with DAPI.
8. Put coverslip with aqueous-based mounting media.

SOLUTIONS

0.1M TEA (100ml)

1.85g TEA
100ml water
450µl 10N NaOH
250µl acetic anhydride

Hybridization solution (100ml; aliquot in 10ml and store at -20C)

50% formamide	50ml	formamide
10% dextran sulphate	20ml	50% dextran sulphate
1% Denhardt's solution	2ml	50x Denhardt's (from -20C)
250µg/ml yeast RNA	1.25ml	20mg/ml yeast RNA
0.3M NaCl	6ml	5M NaCl
20mM Tris-HCl, pH 8	2ml	1M Tris
5mM EDTA, pH 8	1ml	0.5M EDTA
10mM NaPO ₄	1ml	1M NaPO ₄
1% sarcosyl	5ml	20% sarcosyl
	11.75ml	sterile water

High Stringency wash (200ml)

50% formamide	100ml formamide
2x SSC	20ml 20x SSC
	80ml water

RNase Buffer (1liter and store at RT)

100ml 5M NaCl
10ml 1M Tris-HCl, pH 7.5
10ml 0.5M EDTA
880ml water

Wash Buffer for BB1 (1xMAB with 0.05% Tween 20)

2xMAB	23.22g	maleic acid (20mM)
(pH 7.5)	17.53g	NaCl (300mM)
	20ml	10N NaOH
	Water up to 1.0 liter	

1% BB1 (Blocking Reagent from BM. Stock of 10% in wash buffer and store at 4C.)

TNT (500ml)

0.1M Tris-HCl, pH 7.5	50ml	1M Tris
0.15M NaCl	15ml	5M NaCl
0.05% Tween 20	250ul	Tween 20