In situ hybridization of DIG-labelled RNA probes

Prepared by Carin Lundmark

Chemicals

Acetic anhydride	SIGMA, A 6404
Albumin, bovine	SIGMA, A-9647
AquaPerm Mounting medium, 290 ml	Immunon, 484990
5-Bromo-4-chloro-3-inolyl-phosphate (BCIP)	B.M., 1585002
Blocking Reagent	B.M., 1 096 176
Dextran sulphate	SIGMA, D 8906
Diethyl pyrocarbonate (DEPC)	SIGMA, D 5758
Dimethylformamide, 100%	BDH, Cat.No. 10322
Ethanol, 95% & 99.5%	Kemetyl
Ficoll	SIGMA, F-2637
Formamide	MERCK, 1.09684
Glycine pa.	MERCK, 1.04201
Hydrochloric acid fuming	MERCK, 1.00317
4-Nitro blue tetrasodium chloride (NBT)	B.M, 1087479
Magnesium chloride hexahydrat pa.	MERCK, 1.05833
Maleic acid for syntesis	MERCK Sch., 800380
Mixed Bed Resin AG, 501-X8 Resin 20-50 mesh	BIORAD
Paraformaldehyde for syntesis	MERCK Sch., 818715
Pertex mounting medium	HISTOLAB Prod., 00811
Potassium chloride pa. (KCl)	MERCK, 4936
Potassium dihydrogen phosphate pa. (KH_2PO_4)	MERCK, 4873

Proteinase K	SIGMA, P-2308
Polyvinyl pyrrolidone (PVP)	SIGMA, P-5288
Ribonuclease A (RNase A)	SIGMA, R-6513
Rabbit anti-DIG-AP, Fab fragments, rabbit F(ab´)	DAKO, D5105
Ribonucleic acid transfer, from Bakers Yeast,Type X	SIGMA, R-9001
Sodium chloride pa. (NaCl)	MERCK, 1.06404
Sodium hydrogen phosphate, anhydrous pa. (Na_2HPO_4)	MERCK, 1.06586
Titriplex III pa. EDTA	MERCK, 1.08418
Triethanolamine pa.	MERCK, 1.08379
Tri-sodiumcitrate dihydrate	MERCK, 1.06448
Tris, Molecular Biology Certified	IBI, IB70142
Triton X-100	SIGMA, X-100
Xylene pa.	MERCK, 1.08681
Digoxigenin(DIG)-labelled mRNA probe (riboprobe), prepared according to Boehringer Mannheim protocol.	

Stock and working solutions

50X Denhardt's Reagent

		Final conc.
500 mg	Ficoll	1%
500 mg	Polyvinylpyrrolidone (PVP)	1%
500 mg	RNase-free bovine serum albumin	10 mg/ml

Add DEPC-H_20 to final volume of 50 ml.

Hybridization buffer

		Final conc.
10 ml	Formamide, deionized	50%
2 ml	3 M NaCl	0.3 M

0.2 ml	2 M Tris-HCl, pH 8	10 mM
0.04 ml	0.5 M EDTA, pH 8	1 mM
1 ml	10 mg/ml tRNA	0.5 mg/ml
4 ml	0.5 g/ml DS	100 mg/ml
0.4 ml	50X Denhardt's reagent	1X
2.36 ml	DEPC-H_2O	

Aliquot in eppendorf tubes. Hybridization buffer can be stored at -20oC for several months.

4% Paraformaldehyde, pH 7.0; 200 ml

8 g	Paraformaldehyde
600 µl	5 M NaOH (in DEPC-H_2O)
20 ml	10X PBS (in DEPC-H_2O)

Triethanolamine-HAc; 200ml

2.66 ml	Triethanolamine
200 ml	DEPC-H_2O

Submerse slides, add 500 ml acetic anhydride. Mix the two solutions by moving slides in the solution.

Prehybridization mixture; 200ml

20 ml	20X SSC (in DEPC-H_2O)
100 ml	Formamide, deionized
80 ml	DEPC-H_2O

Hybridization solution

Add riboprobe to hybridization buffer: 0.5 ng probe/µl . Place the solution for 3 min on heating block 92-95°C.
Cool immediately on ice.
100 µl hybridization solution/glass is applied per glass
Hybridization solution is prepared freshly just before the hybridization step.

Buffer 1; 800 ml

		Final conc.
80 ml	1 M Tris-HCl, pH 7,5	100 mM
40 ml	3 M NaCl	150 mM

Add autoclaved, deionized water to final volume of 800 ml.

Buffer 1 with Triton; 800 ml

10 ml Buffer 1
800 ul Triton X-100

Add Triton X-100 on the inner wall of the test tube and dilute with Buffer 1.
Leave solution on a shaking platform for a few minutes.

RNase buffer; 600 ml

		Final conc.
100 ml	3 M NaCl	500 mM
6 ml	1 M Tris-HCl, pH 8	10 mM
1.2 ml	0.5 M EDTA, pH 8	1 mM

Add autoclaved, deionized water to final volume of 600 ml.

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20 mg/ml RNase; 150 ml

3 mg RNase A
150 ml RNase buffer

Antibody solution; 3 ml
Dilute the Anti-DIG-AP antibody 1:1000 in Buffer 1 with Triton. Apply 100 µl/glass.

Buffer 2; 200 ml

		Final conc.
20 ml	1 M Tris-HCl, pH 7.5	100 mM
10 ml	3 M NaCl	150 mM
200 ml	Triton X-100	0.1 %
20 ml	10% Blocking Reagent	1 %

Add autoclaved, deionized water to final volume of 200 ml.

Buffer 3; 200 ml

		Final conc.
20 ml	1M Tris-HCl, pH, 9.5	100 mM
5 ml	4 M NaCl	100 mM
10 ml	1 M $MgCl_2$	50 mM

Add autoclaved, deionized water to final volume of 200 ml.

Substrate solution; 3 ml

3 ml	Buffer 3
13.5 µl	NBT
10.5 µl	BCIP

TE 8; 200 ml

		Final conc.
1 ml	2 M Tris-HCl, pH 8	10 mM
400 ml	0.5 M EDTA, pH 8	1 mM

Add autoclaved, deionized water to 200 ml.

Paraffin sections protocol:

Day 1

1. Xylene	2 x 5 min	

2. abs EtOH	Rinse	
3. abs EtOH	5 min	
4. 95% EtOH	Rinse	
5. 70% EtOH	Rinse	
6. 1X PBS	Rinse	
7. 0.2M HCl	15 min	
8. 1X PBS	2 x 5 min	
9. 20 μg/ml Proteinase K	30 min, 37°C	Use humid box.
10. 0.2% Glycine	Rinse	
11. 4% Paraformaldehyde	5 min	
12. Triethanolamine. When slides are in the dish: Add 500 μl acetic anhydride. Mix solutions by dipping slides several times.	10 min	Shaking platform
13. 1X PBS	Rinse	
14. 1X PBS	5 min	
15. Prehybridization mixture	60 min, 42°C	Water bath
16. Hybridization solution	Over night, 42°C	Draw a fat-ring around the section. Apply 100 μl/glass. Use humid box.

Day 2

17. 4X SSC	Rinse	Rinse each probe separately
18. 2X SSC	2 x15 min, 37°C	Oven with shake board
19. 1X SSC	2 x15 min, 37°C	Oven with shake board
20. 20 μg/ml RNase	30 min, 37°C	Special oven
21. RNase buffer	Rinse, 37°C	
22. RNase buffer	5 min, 37°C	Special oven
23. 2X SSC, 50% formamide	2 x 30 min, 52°C	Oven with shake board
24. Buffer 1	Rinse	
25. Buffer 1	5 min	
26. Buffer 2	30 min	Shaking platform.
27. Antibody solution	30 min, 37°C	Oven. Apply 100 μl/glass.
28. Buffer 1	Rinse	

29. Buffer 1	5 min	
30. Buffer 3	2 min	
31. Substrate solution	15 min to 3 hours (in the dark).until desired colour has eveloped. Examine sample by microscope.	Apply 100 μl/glass.
32. TE8	Rinse	

33. Mount sections with AquaPerm. Apply 1-3 drops of AquaPerm over the section.
Leave section to dry: 1 hour at room temperature or 20-30 minutes at 40-60°C in oven.
Make sure that the surface is completly dry and hard before you mount and coverslip sections with Pertex.

Cryo-sections protocol:

Day 1

1. 95% EtOH	5 min	
2. 70% EtOH	Rinse	
3. 1X PBS	2 x 5 min	
4. 20 μg/ml Proteinase K	30 min, room temp	Humid box
10. 0.2% Glycine	Rinse	
11. 4% Paraformaldehyde	5 min	
12. Triethanolamine. When slides are in the dish: Add 500 μl acetic anhydride. Mix solutions by dipping slides several times	10 min	Shaking platform
13. 1X PBS	Rinse	
14. 1X PBS	5 min	
15. Prehybridization mixture	60 min, 42°C	Water bath
		Draw a fat-ring around the section
16. Hybridization solution	Over night, 42°C	Apply 100 μl/glass. Use humid box.

Day 2

17. 4X SSC	Rinse	Rinse each probe separately.
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20. 20 μg/ml RNase	30 min, 37°C	Special oven
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22. RNase buffer	5 min, 37°C	Special oven
23. 2X SSC, 50% formamide	2 x 30 min, 52°C	Oven with shake board
24. Buffer 1	Rinse	
25. Buffer 1	5 min	
26. Buffer 2	30 min	Shaking platform
27. Antibody solution	30 min, 37°C	Oven. Apply 100 μl/glass.
28. Buffer 1	Rinse	
29. Buffer 1	5 min	
30. Buffer 3	2 min	
31. Substrate solution	15 min to 3 hours (in the dark) until desired colour has developed. Examine sample by microscope.	Apply 100 μl/glass.
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Tissue preparations

Fixation
Keep the time between killing of animal and fixation as short as possible, for best preservation of morphology.
Place tissue in freshly prepared paraformaldehyde, 4%.
Fix the tissue over night (not more than 24 hours) at 4°C.

Decalcification
Discard paraformaldehyde solution. (Rinsing tissue is not necessary.)
Cover the tissue with EDTA-solution (~12.5%, pH 7) for decalcification.
Keep at 4°C with gentle shaking.
Change EDTA-solution every 3-4 days.
Check end-point with x-ray.
After decalcification: Rinse the samples in 1X PBS, some hours to over night, on shaking platform.

Dehydration and paraffin infiltration
Use Vacuum Infiltration Processor (V.I.P.). Program 1 is suitable fo rat jaws.
Keep blocks in refrigerator until use. Long storage times up to years are possible.

Freezing tissue

After completed decalcification , leave tissue in 10% sucrose over night. Freeze the tissue in liquid nitrogen or hexane on dry ice. Store in -70°C freezer.

Sectioning

Paraffin

Wear gloves during sectioning procedures.
Cut 7 μm thick sections from the paraffin-embedded material.
The water bath should contain DEPC-$H_2$0.
Place sections on silan-coated microscope slides.
Leave sections to dry at 56°C in oven over night.
Keep section in refrigerator until use; for maximally 1 month.

Cryo

Mount tissue on a prepholder, use cryomount to stabilize the tissue.
Cut sections at 7μm. Mount section on silan coated slides.
Leave section to dry in 56°C over night. Store sections at 4°C until use.