

***In situ* hybridization**

References; Schaeren-Wiemers and Gerfin-Moser, 1993, *Histochemistry* **100**: 431-440.
Giger *et al.*, 1996, *J. Comp. Neurol.* **375**: 378-392.
Pasterkamp *et al.*, 1999, *Mol. Cell. Neurosci* **13**: 143-166.

FIXATION (room temperature)

Wear **Gloves!!!**

Perfused tissue:

- 5 min fixation in 4% Para formaldehyde in PBS (pH 7.4).
- rinse in PBS.
- 10 min incubation in 10 μ g/ml proteinase K in PBS (optional 0.1% Triton X-100).
- 10 min fixation in 4% Para formaldehyde in PBS.
- Follow protocol from PBS washes*

Fresh tissue:

- 20 min fixation in 4% Para formaldehyde in PBS (pH 7.4).
- Follow protocol from PBS washes*

-5 min wash in PBS (pH 7.4)*.

-5 min wash in PBS.

-5 min wash in PBS.

-Rinse in milliQ-H₂O.

-10 min acetylation in 0.25% acetic anhydride in TEA;

-dissolve 1 ml triethanolamine in 100 ml milliQ-H₂O (TEA).

-immerse slides in TEA and immediately apply 250 μ l acetic anhydride. Stir.

-5 min wash in PBS.

-5 min wash in PBS.

-5 min wash in 2x SSC.

PREHYBRIDIZATION (room temperature)

-2 h till overnight incubation in hybridization solution (150 μ l/slide) covered with Nescofilm in a humidified chamber (H₂O). Use a DAKO pen.

HYBRIDIZATION (60°C)

- Add 200 ng (or do serial dilution)/ml cRNA probe (sense or antisense) to hybridization solution on ice (= hybridization mix).
- Denature hybridization mix for 5 min at 85 °C.
- Put the mix 5 min on ice.
- Pour off the hybridization solution (reuse Nescofilm) and add 150 μ l hybridization mix per slide.
- Hybridize slides overnight at 60 °C covered with Nescofilm in a humidified chamber.

STRINGENCY WASH (60 °C, room temperature)

- 5 min wash in 5x SSC at 60 °C.

- 1 min wash in 2x SSC at 60 °C.
- 30 min wash in 0.2x SSC in 50% formamide (reuse this solution) at 60 °C.
- 5 min wash in 0.2x SSC at room temperature.

IMMUNOLOGICAL DETECTION (room temperature)

- 5 min wash in buffer 1.
- 1 h block in 1% blocking reagent in buffer 1 (heat to 60 °C until dissolved and cool down before use).
- 5 min wash in buffer 1.
- 1.5-3 h antibody incubation (1: 3000 anti-DIG-AP Fab in buffer 1 (400 ul/slide) in a humidified chamber.
- 15 min wash in buffer 1.
- 15 min wash in buffer 1.
- 5 min wash in buffer 2.

COLOUR REACTION (room temperature, darkness)

- Light sensitive color reaction 1 h- 48 h(400 ul/slide) in a humidified chamber.
- To stop reaction wash for 10 min in TE.
- Dehydrate and mount in Entellan or immediately in aqueous mounting media (glycerol).

BUFFERS

Hybridization solution (store at – 20°C) (Final concentration)

5 ml 50x Denhardt's	(5x)
<ul style="list-style-type: none">• 5 g Ficoll, 5 g polyvinylpyrrolidone and• 5 g bovine serum albumin in 500 ml H₂O.• Filter and store at – 20°C.	
125 ul 100 mg/ml baker yeast tRNA	(205 ug/ml)
1 ml 10 mg/ml herring sperm DNA (optional)	(200ug/ml)
12.5 ml 20x SCC	(5x)
25 ml form amide	(50%)
Fill up to 50 ml with DEPC- H ₂ O (= 1 ml DEPC in 1 L H ₂ O, Stir for 2 h, then autoclave).	

10x Phosphate Buffered Saline (PBS) DEPC!!

12.7 g Na ₂ HPO ₄	(9 mM)
2.65 g NaH ₂ PO ₄ ·2H ₂ O	(1.7 mM)
85 g NaCl	
Add H ₂ O, adjust pH to 7.4, fill up to 1 liter, treat with DEPC and autoclave.	

20x SSC DEPC!!

175.3 g NaCL

88.2 g sodium citrate

Add H₂O, adjust pH to 7.0, fill up to 1 liter, treat with DEPC and autoclave.**Buffer 1(1x) NO DEPC!!**

100 ml 1 M Tris (100 mM)

12.1 g/l

30ml 5 M NaCL (150 mM) 8.7

g/l

Add millQ-H₂O; adjust pH to 7.5, fill up to 1 liter. Make this buffer fresh before use.**Buffer 2(1x) NO DEPC!!**

100 ml 1 M Tris (100 mM)

12.1 g/l

20 ml 5 M NaCL (100 mM)

5.84 g/l

5 ml 1 M MgCL₂ (5mM)Add millQ-H₂O; adjust pH to 9.5, fill up to 1 liter. Make this buffer fresh before use.**1 M MgCL₂**60.99 g MgCL₂.6 H₂O in 300 ml H₂O. Autoclave. (1M)**10x TE NO DEPC!!**

100 ml 1 M Tris (100 mM)

12.1 g/l

20 ml 0.5 M EDTA (10 mM) 3.72

g/l

Add H₂O, adjust pH to 8.0, fill up to 1 liter and autoclave.**COLOUR SOLUTION *Light sensitive!!***

45 ul 75 mg/ml NBT (in 70% dimethylformamide)

35 ul 50 mg/ml BCIP-phosphate (in 100% dimethylformamide)

2.4 mg Levamisole

In 10 ml buffer 2. Filter solution through a 0.22 um filter and store in darkness until use.

CHEMICALS

	NL	USA
Acetic acid (96%) A6283	Merck, 251	<i>Sigma,</i>
Acetic anhydride A6404	Merck, 42	<i>Sigma,</i>
Anti-digoxigenin-AP-Fab fragments	Roche, 1093274	

Biophenol	Biosolve, 1690Q403
<i>Corestore 15513039</i>	
Bovine serum albumin	Sigma, A-4503
<i>Amersham, 70 195</i>	
Blocking reagent	Roche, 109617
Chloroform GR	Merck, 102445
<i>JT.Baker 9180-01</i>	
DAKOpen	DAKO, s200230
Diethyl pyrocarbonate	Sigma, D-5758
DIG labeling mix (10x)	Roche, 1277073
Dimethyl formamide	Analar, 10322
di-Sodium hydrogen phosphate anhydrous	Merck, 106586
<i>JT.Baker 3828-1</i>	
Dry acetone (99.5% pure)	Lamers&Pleugers,
0034010003	
Dithiothreitol	Roche, 197777
<i>Sigma D9779</i>	
Ethanol (ethyl alcohol)	Merck, 100983
(Ethylenedinitrilo)tetraacetic acid disodium salt	Roche, 808288
<i>JT.Baker, 8993-1</i>	
Ficoll 400	Pharmacia, 17040001 <i>Sigma,</i>
<i>F4375</i>	
5-bromo-4-chloro-3-indolyl phosphate	Roche, 1585002
Formamide	Fluka-Chemika, 47670,
<i>Fisher, BP227500</i>	
Herring sperm DNA	Roche, 223646
Isoamylalcohol	<i>JT</i>
<i>Baker, 9038-01</i>	
Isopentane (2-methylbutane)	<i>JT</i>
<i>Baker, Q223-07</i>	
Levamisole	Sigma, L-9756
Lithium Chloride	Sigma, L-9650
Magnesiumchlorid-hexahydrate	Merck, 5833
<i>JT.Baker 9044-3</i>	
Milli Q water	Millipore, 18.2 ohm
4-Nitrobluetetrazolium chloride	Roche, 1585029
Nescofilm	Roth, 2569
Paraformaldehyde (extra pure)	Merck, 104005 <i>Sigma,</i>
<i>P6148</i>	
Polyvinylpyrrolidone	Merck, 7443 <i>Sigma,</i>
<i>P6755</i>	
Proteinase K	Roche, 1000144
Restriction enzymes	Roche
<i>Biolabs</i>	
RNAse inhibitor	Roche, 799017
RNA polymerase T3	Roche, 1031169

RNA polymerase T7	Roche, 881767	
RNA polymerase SP6	Roche, 810274	
Sodium acetate tri-hydrate <i>BP, 334500</i>	Merck, 106267	<i>Fisher</i>
Sodium carbonate anhydrous <i>Baker, 3604-1</i>	Merck, 6392	<i>JT</i>
Sodium chloride <i>BP 3582</i>	Merck, 101540	Fisher
Sodium citrate dihydrate <i>Baker, 3646-1</i>	Merck, 106448	<i>JT</i>
Sodium dihydrogen phosphate dihydrate (extra pure) <i>JT.Baker, 3819-01</i>	Merck, 6345	
Sodium hydrogen carbonate <i>S-6014</i>	Merck, 6329	<i>Sigma,</i>
Sodium hydroxide (pellets)	Merck, 106498	
Superfrost slides <i>12-550-15</i>	Menzel, 3672286	<i>Fisher,</i>
3-Aminopropyltriethoxysilane	Sigma, A-3648	
Transcription buffer (10x)	Comes with polymerases	
TRNA	Roche, 109509	
Triethanolamine (extra pure) <i>T-1377</i>	Merck, 8377	<i>Sigma,</i>
Tris(hydroxymethyl)-aminomethane (ultra pure) <i>6791</i>	Biosolve, 56264	<i>Sigma,</i>
Triton X-100	Sigma, X-100	