IN SITU HYBRIDIZATION PROTOCOL

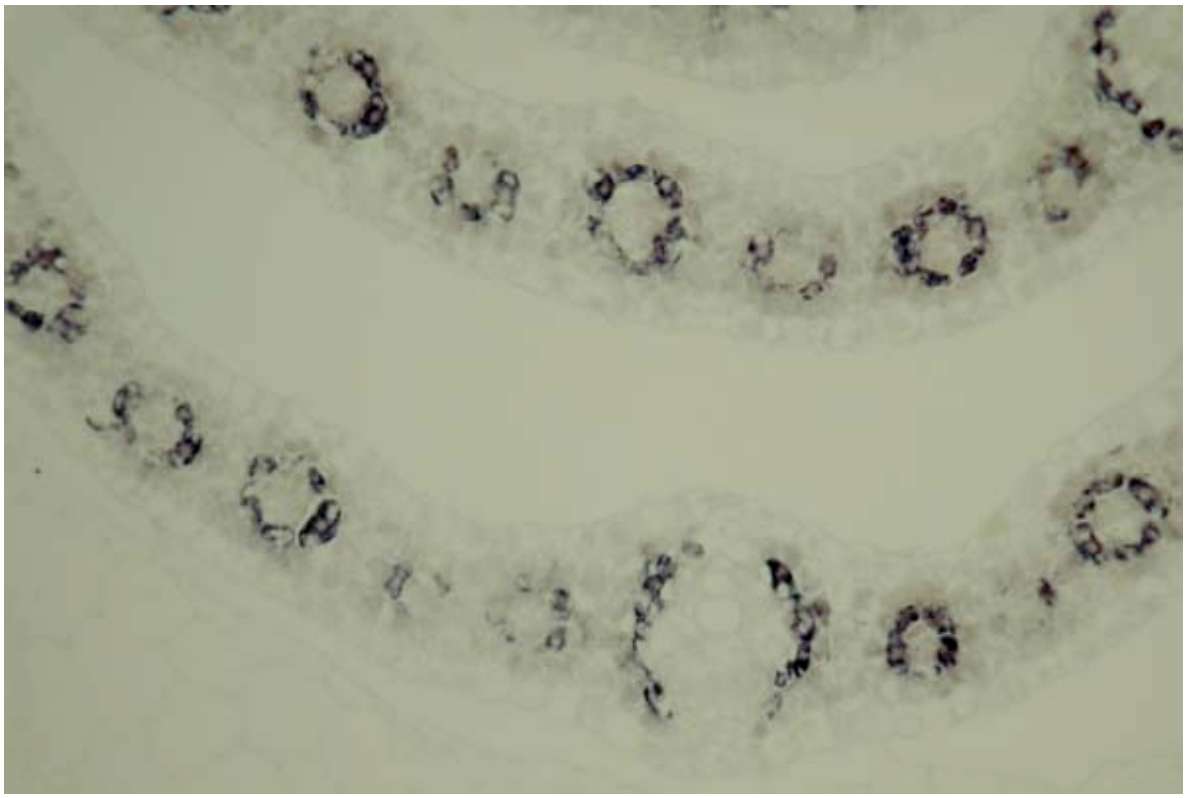
..... as used in the *Antirrhinum* labs of the Genetics Department, John Innes Centre, Colney Lane, Norwich January 1997

..... modifications by Jane Langdale, Dept of Plant Sciences, University of Oxford, South Parks Rd, Oxford March 2001

REFERENCES

Jackson, D.P. (1992). *In situ* hybridization in plants. In Molecular Plant Pathology: A Practical Approach (D.J. Bowles, S.J. Gurr and M.McPhereson, Eds) Oxford University Press, Oxford, pp. 163-174.

Langdale, J.A. (1993) *In situ* hybridization. In: The Maize Handbook. (V. Walbot and M. Freeling, Eds) Springer-Verlag, New York, pp165-180



TISSUE FIXATION AND EMBEDDING

This is a very critical step; badly fixed material will give little or no *in situ* signal even with probes against highly abundant messages.

Choice of Fixative

We recommend the use of 4% (w/v) formaldehyde with 0.1% tween-20 and 0.1% triton-x-100. Formaldehyde vapour is toxic, thus solutions should be handled in the fume hood. In addition, due to instability, solutions should be freshly prepared from paraformaldehyde just before use, or freeze/thawed only once. FAA (50% ethanol : 10% formalin : 5% acetic acid) also works just as well but 3 : 1 ethanol : acetic acid gives no signal!

Size of tissue

In general, the smaller the better. Tissue should be placed in fix immediately after dissection, thus if you cannot bring the plant to the fume hood, cut large pieces of tissue and transport on ice, then cut into smaller pieces at the fume hood. Ideally, one dimension of the tissue should be <2mm and the other <10mm.

Penetration of fixative

Most plant tissues have a cuticle and will thus simply float on the surface of the fixative. To allow penetration of the fixative, the tissue must be vacuum infiltrated. To do this, sometimes you have to weigh down the tissue under the surface, eg. by using a piece of wire guaze, though the use of detergents often avoids this problem. Place sample in a vacuum desiccator and apply a vacuum using an oil pump (a bench type water pump does not provide adequate suction). Care should be taken to ensure the solution does not boil under the reduced pressure. Formaldehyde vapour is volatile, so the fixative must be replaced after the vacuum treatment.

Fixation/Embedding Protocol

The original protocol had 0.85% saline in the ethanol series. This is not necessary either for tissue integrity or hybridization signal.

Day 1

4% formaldehyde, 0.1% Tween-20, 0.1% triton OVERNIGHT on ice or 4°C.

Day 2

30% Ethanol	1 Hr	on ice
40% Ethanol	1 Hr	on ice
50% Ethanol	1 Hr	on ice
60% Ethanol	1 Hr	on ice
70% Ethanol	1 Hr	on ice
85% Ethanol	1 Hr	on ice
95% Ethanol + 0.1%Eosin	OVERNIGHT	4°C

Day 3

100% Ethanol	30 min	room temp
100% Ethanol	30 min	room temp
100% Ethanol	1 Hr	room temp
100% Ethanol	1 Hr	room temp
25% Histoclear/75% Ethanol	1 Hr	room temp
50% Histoclear/50% Ethanol	1 Hr	room temp
75% Histoclear/25% Ethanol	1 Hr	room temp
100% Histoclear	1 Hr	room temp
100% Histoclear	1 Hr	room temp
100% Histoclear/1/4 vol wax	OVERNIGHT	room temp

Day 4

Place at 42°C until wax melts completely and then add 1/4 volume fresh chips. Keep at 42°C until wax melts and then move to 60°C for several hours. Replace

with fresh wax overnight at 60°C.

Day 5-7

Replace with 100% wax and change twice daily. Pour wax slowly to avoid bubbles.

Day 8

Make the tissue blocks in peel-away molds (Polysciences Inc. Cat #18985 or 18646A). Pour a layer of wax sufficient to cover the tissue and orientate the tissue in the mold. Remove from the heated block and as wax just begins to solidify, float mold on water. After the surface begins to solidify and indent, submerge the mold and leave 2-10 min to solidify completely. Store the blocks at 4°C until use.

Preparation of slides and cover slips

Pre-coated slides can be bought from BDH (Cat. No. 406/0178/00).

To prepare cover slips, wash in acetone for 15 min. and bake overnight at 80°C.

Sectioning

Good sectioning requires lots of practise (patience!). It is a good idea to spend a few hours with someone who is skilled in wax sectioning.

1. Cut the wax block to a trapezoid shape, leaving about 2mm of wax around the tissue (more than this will not aid sectioning, and will give fewer sections per slide).
2. Mount the block such that the longer of the two parallel faces is at the bottom (ie. is the first to strike the blade).
3. Cut ribbons of section at 7-10 μm thickness. Float onto sterile water on coated slides then place on 42°C hotplate for a few minutes until ribbon flattens out. Drain off the excess water, and then press with lens tissue.
4. Leave on the hotplate overnight to dry.

Common problems with sectioning:

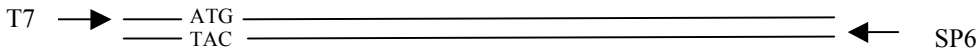
- Sections break up - material not properly embedded
- Sections split along the ribbon - blade chipped or dirty
- Ribbon not straight - faces of block not parallel to the blade
- Sections roll up rather than forming a ribbon - angle of blade is wrong or the blade needs cleaning with 100% ethanol

DIGOXIGENIN LABELLING PROBES.

DO NOT USE THE BOEHRINGER/ROCHE DIG MIX TO LABEL PROBES. Use of the mix significantly increases background as compared to using the protocol below. The method below is just as efficient ion terms of labelling and intensity of hybridisation as methods that use 50:50 labelled : unlabelled UTP. Although the latter methods make more RNA, it is not as well labelled and thus sensitivity of hybridisation is lower. This protocol is also much cheaper!

Template preparation

The template should consist of a 150-1200 bp insert (without any poly A tail) in any high copy vector such as Bluescript in front of the T7 or T3 promoter (we found the SP6 promoter was variable – but it does work OK most of the time). Optimal templates have about a 500 bp insert. Template DNA should not be made by miniprep methods as RNAase contamination limits the amount of probe recovered. **Reminder:** In example below, T7 will make sense probe and SP6 will make antisense.



1. Prepare plasmid using Qiagen midi or maxi kit.

2. Linearize plasmid in two separate reactions, at 5' and 3' end of insert. Do not use enzymes that leave a 3' overhang, as the RNA polymerase efficiency will be reduced in subsequent reactions.

3. Phenol/chloroform extract and ethanol precipitate as normal. Resuspend in DEPC treated water at 1mgml^{-1}.

Probe Synthesis

1. Set up reactions on the bench (not on ice because the RNA polymerase buffer forms precipitates. Also make sure that the precipitates in the

buffer are fully dissolved after thawing).

Reaction mix:

9.5 µl dH_20

2.5 µl 10 X RNA polymerase buffer

1 µl RNAse inhibitor

2.5 µl 5mM ATP

2.5 µl 5mM GTP

2.5 µl 5mM CTP

2.5 µl 1mM DIG-UTP

1 µl linearized DNA template (ca. 0.5-1 µg) (T7/T3/Sp6 work on ds DNA)

1 µl T7 or T3 polymerase

2. Incubate for 40 min. at 37°C

3. To stop reaction add: 75 µl 1 X MS (see stock solutions)
2 µl tRNA (100 mg/ml)
1 µl DNase (RNase free)

4 . Incubate for 10 min. at 37°C

5. Precipitate with: 50 µl 7.5M NH_4Ac*
400 µl EtOH (ice cold)

* If you use NaOAc here instead of NH_4Ac, you will lose a lot of RNA during the precipitation

6. -20°C overnight (or 10 min. on dry ice).

7. Spin down 10 min. at 4°C at top speed in a microfuge (13000-15000 rpm)

8. Wash in 70% ethanol and spin again

9. Remove supernate and air dry (or speedvac)

10. Resuspend in 50 μl dH_20 (sterile)

11. Add 50 μl 200mM carbonate buffer pH 10.2

12. Hydrolyse at 60°C:*

60 min	for	insert/templates of	400-500 bp
20-30 min.	"	"	200-400 bp
1-5 min	"	"	<200 bp
80-90 min.	"	"	600-1200 bp

* A formula can be used to find the exact time needed to generate the 150-200 bp fragments that are optimal, but we find that these empirical times work well.

13. Precipitate with:
    - 10 μl 10% HAc (keep frozen)
    - 12 μl 3M NaAc (pH 4.8)
    - 312 μl Et0H (ice cold)

14. -80°C for 1-2 hr

15. Spin down, wash and dry as above.

16. Resuspend in 50 μl DEPC treated H_20

Check of labelling efficiency

1. Remove 2 μl of probe to a fresh tube and add 48μl formamide to the remainder. Store probe/50% formamide at -80°C for up to twelve months.
2. Spot 1μl of probe plus 1μl of 1/10th and 1/100th dilutions onto a piece of nylon membrane, alongside identical dilutions of the Roche control labelled RNA
3. UV X-link filter
4. Wet filter in DIG Buffer 1 (see stock solutions) in a small petri dish
5. Incubate for 30 min. in DIG Buffer 2.
6. Wash 2-3 times in Buffer 1 (5 min. each).
7. React for 30 min. in 5 ml Buffer 1 plus 1 μl anti-DIG antibody
8. Wash 2 x 15 min. in Buffer 1
9. Wash briefly in DIG Buffer 5
10. Incubate in DIG Buffer 6 (5 ml Buffer 5 plus 7μl NBT stock and 5 μl BCIP stock) until all of the control probe samples are visible
11. If labelling is optimal, intensity of signal should be same as control probe.

IN SITU HYBRIDIZATION

The night before:

- Autoclave 8L of distilled water for each rack of 24 slides.
- Bake acetone-treated cover slips (can be done before this).
- Check stock solutions (see end)
- Soak a 500 ml glass graduated cylinder in distilled water with 2-3 NaOH pellets
- Make sure you have a supply of sterilized eppendorfs and tips
- Book a 50°C oven for next overnight
- Bake slide racks

First day:

Tissue pretreatment

1. Prepare the following solutions (start with the paraformaldehyde) using the glass cylinder rinsed with the autoclaved water. Use autoclaved distilled water for all solutions before RNase treatment. For measuring volumes smaller than 50 ml use sterile orange cap tubes or disposable pipettes. The volumes are sufficient for two racks of slides. Put the solutions in troughs:

- **Histoclear 1** and **2** (glass troughs in fume hood, 2x350 ml)

- **100% ethanol 1** and **2** (1 - glass trough in fume hood, 2 - plastic trough on the bench, 2x350 ml)

	100% EtOH	8.5% NaCl	H_2O
• **95% ethanol** (350 ml):	333 ml	-	17 ml
• **85% ethanol, 0.85% NaCl** (350 ml):	298 ml	35 ml	17 ml
• **50% ethanol, 0.85% NaCl** (350 ml):	175 ml	35 ml	140 ml
• **30% ethanol, 0.85% NaCl** (350 ml):	105 ml	35 ml	210 ml
• **0.85% NaCl** (350 ml):	-	35 ml	315 ml

- **PBS 1 and 2** (350 ml each):

10X PBS	2 x 35 ml
H_2O	2 x 315 ml

- **Pronase 0.125 mgml^{-1}** (350 ml):

20 x Pronase buffer	17.5 ml
H_2O	331.5 ml
Pronase stock 40 mgml^{-1} (freezer)	1 ml

- **Glycine 0.2%** in PBS (350 ml):

10X PBS	35 ml
H_2O	309 ml
Glycine stock 10% (fridge)	6 ml

- **4% paraformaldehyde** in PBS (350 ml):

10x PBS	35 ml
H_2O	315 ml
Paraformaldehyde (cold room)	12 g

*Prepare PBS using the graduated cylinder, pour into a glass beaker, add 2-3 pellets of Na0H and heat on a stirring hotplate to 60°C. Weigh paraformaldehyde in fume hood, add to the PBS and stir until dissolved. Adjust pH to 7.0 with HCl (check with pH paper). Be careful, the pH changes very abruptly! Paraformaldehyde is very toxic, always seal well or keep in fume hood.

- **Acetic anhydride** in 0.1M triethanolamine pH8 (400 ml):

Triethanolamine	5.2 ml
HCl	1.6 ml
H_20	391.2ml
Acetic anhydride	2 ml

* Place the trough on a magnetic stirrer in the fume hood with an empty rack or cut off pipettes at the bottom. Place a stir bar in the centre of the empty rack or between the pipettes. Stir the solution and add the acetic anhydride after placing the rack of slides in the trough. Acetic anhydride is very unstable in water so add fresh triethanolamine buffer and acetic anhydride must be used if you have a second rack of slides.

2. Place slides in stainless steel racks and pass through the solutions in the following order:

fumehood	Histoclear 1 (**)	10′
fumehood	Histoclear 2	10′
fumehood	100% ethanol 1	1′
	100% ethanol 2	30″
	95% ethanol (***)	30″
	85% ethanol, 0.85% saline	30″
	50% ethanol, 0.85% saline	30″
	30% ethanol 0.85% saline	30″
	0.85% saline	2′
	PBS 1	2′
	Pronase	10′ 37°C
	Glycine	2′
	PBS 1	2′
fumehood	Paraformaldehyde	10′
fumehood	PBS 1	2′
fumehood	PBS 2	2′

fumehood	Acetic anhydride	10′
fumehood	PBS 2	2′
	0.85% saline	2′

** For the second rack, Histoclear 2 will be Histoclear 1. Discard the former Histoclear 1 and fill with fresh histoclear which will be Histoclear 2 in the next series.

***The ethanol series can be reused.

****Rinse out the troughs with distilled water, allow to dry and store with a little ethanol in each.

3. Dehydrate through the ethanol series (the reverse of the above 30% up to 100%), wash in fresh 100% ethanol. Wrap in paper towel and leave on bench to dry.

Hybridization

1. Prepare hybridization buffer (32 μl per slide with 22 x 50 mm coverslip) *

Hybridization buffer

	24 slides	**48 slides**
10 x salts	100 μl	200 μl
deionized formamide (freezer)	400 μl	800 μl
tRNA 100 $mgml^{-1}$	10 μl	20 μl
50 x Denhardt's	20 μl	40 μl
H_20	70 μl	140 μl
50% dextran sulphate** (freezer)	200 μl	400 μl
final volume	800 μl	1600 μl

* When 3 or more slides use the same probe, make the mix for 1 slide more than the actual number

**The dextran sulphate is very viscous, add it at the end using a cut-off blue tip.

2. Vortex the hybridization buffer, spin down and leave at room temperature.

3. Take the slides out of the rack, allow ethanol to evaporate completely.

4. Heat the probe/50% formamide mix for 2′ at 80°C, spin down and cool on ice.

5. Mix the probe with the hybridization buffer in a 4:1 buffer : probe ratio*. If using two probes from different regions of the gene, mix in a 4 : 0.5 : 0.5 ratio. Vortex, spin down and leave at room temperature.

* If you use a higher concentration of probe, you will just increase the background relative to the signal.

6. Draw around sections with a Pap pen (Sigma Z37,782-1) and then add buffer/probe to slide.

7. Lower coverslip onto slide, avoiding bubbles

8. Place paper towels at the bottom of sandwich boxes and soak with distilled water. (You do not need to soak in formamide). Lay cut off plastic pipettes on top of the towels and lay slides on tramlines created by pairs of pipettes. Seal box and place in 50°C oven overnight.

Second day:
It is best to carry out washing and antibody staining on the same day (12 h).

Washing

1. Prepare wash buffer. The original protocol used 2 x SSC, 50% formamide. If you want to avoid using formamide the equivalent is 0.03 x SSC. I use 0.1 x SSC mainly because 0.03 x sounds so low I am afraid I'll lose signal (irrational fear but nevertheless…).

2. Place 2 x 350 ml wash buffer in troughs and into one trough place a slide rack. Dip the slides one at a time into the first trough and agitate gently until the coverslips fall off. Once the coverslips are removed, place the slides in the rack in the second trough.

3. Incubate at 50°C for 30' (without shaking).

5. Change wash buffer and incubate for a further 1 h 30' at 50°C.

6. During the wash prepare the following buffer:

   - **NTE** (6 x 350 ml/rack)

	24 slides	**48 slides**
10x NTE	210 ml	420 ml
H_2O	1890 ml	3780 ml

7. Wash in NTE 2 x 5' at 37°C

8. Incubate in NTE containing 20 μg/ml RNase A 30' at 37°C

	24 slides	**48 slides**
RNase A stock 10 $mgml^{-1}$ (freezer)	0.7 ml	1.4 ml
NTE	350 ml	700 ml

*Previously preincubate the NTE-RNase A at 37°C for 5'.

9.	Wash in:	NTE	3 x 5'	37°C
		Wash buffer	1 h	50°C
		PBS	5'	RT

*At this step you can leave the slides in fresh PBS at 4'C overnight but signal will be less intense than if you carry straight through.

Antibody Staining

1. Prepare the following solutions:

- **Buffer 1,** 100 mM Tris-HCl, 150 mM NaCl (2.5L/24 slides)

	24 slides	48 slides
10 x Buffer 1	250 ml	500 ml
H_2O	2250 ml	4500 ml

- **Buffer 2,** 0.5% (w/v) blocking reagent in buffer 1 (100 ml/24 slides)

	24 slides	48 slides
Blocking reagent (Boehringer, 1096 176, kit box- freezer)	0.5 g	1.0 g
Buffer 1	100 ml	200 ml

*Make fresh, let dissolve for 1 hour at 60-70°C, the solution will remain turbid. Cool before use.

- **Buffer 3,** 1% BSA, 0.3% Triton X-100 in Buffer 1 (1.6L/24 slides)

	24 slides	48 slides
BSA (Fraction V)	16g	32g
Buffer 1	1600ml	3200 ml
Triton X-100	4.8 ml	9.6 ml

*Make fresh daily

- **Buffer 4**, Anti-digoxigenin-AP 1:3000 in Buffer 3 (50 ml/24 slides)

	24 slides	48 slides
Anti-digoxigenin-AP (fridge)	17 µl	34 µl
Buffer 3	50 ml	100 ml

*Make fresh shortly before use

- **Buffer 5**, 100 mMTris pH9.5, 100mM NaCl, 50 mM $MgCl_2$ (400 ml/24 slides)

	24 slides	48 slides
10 x Buffer 5A	40 ml	80 ml
10 x Buffer 5B	40 ml	80 ml
H_2O	320 ml	640 ml

- **Buffer 6**, (100 ml/24 slides)

	24 slides	48 slides
Buffer 5	100 ml	200 ml
NBT 75 $mgml^{-1}$ (freezer)	200 µl	400 µl
BCIP 50 $mgml^{-1}$ (freezer)	150 µl	300 µl
Levamisole (24$mgml^{-1}$) (freezer)**	100 µl	200 µl

* Prepare just before use.

** I found that levamisole reduced the background slightly and since it is cheap and easy to make I include it routinely. (I also tried polyvinylalcohol which was a nightmare to work with and made no real difference to background levels).

2. Incubate the slides for 5' in Buffer 1 in a rack in a trough.

3. Transfer the slides to square petri dishes, flood with Buffer 2 and incubate for 30' on a rocking platform

4. Transfer slides back to a rack and incubate for 30' in Buffer 3.

5. Transfer slides back to petri dishes and incubate in Buffer 4 for 1 h 30' on a rocking platform.

6. Transfer slides back to racks and wash in:

Buffer 3	4 x 20'
Buffer 1	5'
Buffer 5	5'

7. Transfer slides back to petri dishes and flood with Buffer 6. Cover the trays with a lid and leave in the dark for 36 h or longer, check after 12 h under a dissecting microscope. Background does not really become a problem until after 72 h.

Third day (if signal is strong, fourth or fifth day if not!):

Washing and counter staining

1. Prepare 0.1% calcofluor (50 ml)

Calcofluor	50 mg
H_2O	50 ml

* Filter the solution using the filter and a syringe.

** Put the solution in a tray and reuse it for all the slides

2.. To stop the enzyme reaction and to wash off background, put slides back in racks and wash for 30 sec. each in troughs containing 350 ml of:

	Ethanol	**H_2O**
distilled H_2O	-	350 ml
70% ethanol	245 ml	105 ml
95% ethanol	333 ml	17 ml
100% ethanol	350 ml	-
95% ethanol	333 ml	17 ml
70% ethanol	245 ml	105 ml
distilled H_2O	-	350 ml

* time of washes will depend on intensity of signal and background; if the background is high, wash for longer

3. Incubate the slides in 0.1% calcofluor 5'

4. Wash briefly in distilled H_2O.

5. Air dry slides in fume hood, add 2-3 drops of Entellan* mounting medium, cover with cover slip of suitable size and dry in the fume hood overnight. The slides are now ready for viewing under UV/visible light microscopy.

* Do not use DPX mountant as the signal will disappear!

** If you want to check signal : noise ratio before mounting permanently, you can mount sections temporarily using 0.5% n-propylgallate in 30% PBS, 70% glycerol or Citifluor. If background is too high you can rinse off mounting medium, wash further in ethanol and then remount.

STOCK SOLUTIONS

- **DEPC treated H_2O**

  Add 1 ml fresh diethylpyrocarbonate (Sigma D-5758) to 1 litre distilled H_2O in fumehood. Shake well, leave overnight at room temperature and then autoclave for 15 min. This water is now RNAase free.

Probe labeling

- **template DNA**

  After restriction digestion, phenol/chloroform extract, ethanol precipitate and resuspend in DEPC treated H_2O at a concentration of 1 $mgml^{-1}$. Store at -20°C.

- **5mM ATP, GTP, CTP**

  Dilute 100 mM stocks (AP Biotech # 27-2025-01) with DEPC-treated H_2O. Store at -20°C.

- **1mM DIG-UTP**

  Dilute Roche stock (#1-209-256) with DEPC-treated H_2O and store at -20°C.

- **10 x RNA polymerase buffer**

  Roche # 1-465-384. Store at -20°C.

- **RNA polymerases (T7, T3 & Sp6)**

  Roche. Store at -20°C.

- **RNAse inhibitor**

  RNAguard - AP Biotech # 27-0815-01. Store at -20°C.

- **MS X 1**

	20 ml
10 mM Tris-HCl pH 7.5	100 µl 2M
10 mM $MgCl_2$	200 µl 1M
50 mM NaCl	666 µl 8.5%

Make 2M Tris by preparing 2M Tris-HCl and 2M Tris-base in DEPC-treated H_2O, and then mixing at a ratio of 8 acid : 2 base to get correct pH. Make 1M $MgCl_2$ and 8.5% NaCl in distilled H_2O and then treat with DEPC as above.

- **tRNA**

  Make tRNA (Sigma #R4251) at 100 $mgml^{-1}$ in DEPC treated H_2O. Store at –20°C.

- **DNAase1**

  Gibco-BRL #18068-015. Store at –20°C.

- **2 x carbonate buffer**

80 mM $NaHCO_3$	0.672 g/100 ml
120 mM Na_2HCO_3	1.277 g/100 ml

  Should be pH 10.2. Add DEPC to 0.1% and treat as above.

- **deionised formamide**

  Sigma # F-9037, aliquot and store at –20°C.

- **DIG labelled control RNA**

  Roche #1585.746. Store at –20°C.

Tissue pretreatment

- **histoclear**

  Fisher #H/0468/17

- **8.5% NaCl**

NaCl	85g

  in lL of dH_2O. Treat with 0.1% DEPC, as above, before use.

- **10 X PBS** (1.5M NaCl, 0.07M Na_2HPO_4, 0.03M NaH_2PO_4 pH 7)

NaCl	85 g
Na_2HPO_4	9.94g
NaH_2PO_4	4.14g

  in lL of dH_2O. Treat with 0.1% DEPC, as above, before use.

- **20 x Pronase Buffer**

	100 ml
1.0M Tris-HCl pH 7.5	50 ml 2M
0.1M EDTA	20 ml 0.5M

Make 2M Tris by preparing 2M Tris-HCl and 2M Tris-base in DEPC-treated H_2O, and then mixing at a ratio of 8 acid : 2 base to get correct pH. Make 0.5 M EDTA pH 8.0 in distilled H_2O and then treat with DEPC as above.

- **pronase**

Sigma # P-6911. Make up to 40 $mgml^{-1}$ in dH_2O. Predigest by incubating for 4h at 37°C. Store at -20°C in l ml aliquots

- **paraformaldehyde**

Sigma #P6148.

- **10% Glycine**

10g glycine in 100 ml of dH_2O. Treat with 0.1% DEPC as above and then store at 4°C.

- **triethanolamine**

Sigma #T-1377.

- **acetic anhydride**

BDH #100022M.

Hybridization

- **10 X salts** (3M NaCl, 0.1MTris-HCl pH 6.8, 0.1M$NaPO_4$ buffer, 50 mM EDTA)

NaCl	8.77 g
2M Tris-Cl pH 6.8	2.5 ml
$NaH_2PO_4.H_2O$	345 mg
Na_2HPO_4	355 mg
0.5M EDTA	5 ml

Combine NaCl and phosphate salts and make up to 42.5ml distilled H_20. DEPC treat as normal and then after autoclaving add 5 ml DEPC-treated 0.5 M EDTA pH 8 and 2.5 ml 2M Tris pH 6.8 made up with DEPC treated H_20 (9.5 acid : 0.5 base).

- **tRNA**

  Make tRNA (Sigma #R4251) to 100mgml^{-1} in DEPC treated H_2O

- **deionised formamide**

  Sigma # F-9037. Aliquot and store at –20°C.

- **50 x denhardt's**

  Sigma #D-2532. Store at –20°C.

- **50% Dextran Sulphate**

  AP Biotech #17-0340-02. Make up with DEPC-treated H_20 and heat in a boiling water bath to dissolve. Store at -20°C in aliquots.

Washes

- **20 X SSC** (3M NaCl, 0.3M Na_3citrate)

NaCl	175.3 g
Na citrate	88.2 g

  up to lL of dH_20. Treat with 0.1% DEPC as above.

- **0.1 x SSC**

20 x SSC	5 ml
DEPC treated H_2O	995 ml

- **10X NTE** (5M NaCl, 100mM Tris-HCl pH 7.5, 10mM EDTA)

NaCl	292.2 g
Tris	50 ml 2 M pH 7.5
EDTA	20 ml 0.5 M pH 8.0

  Make up to in lL in dH_20.

- **RNase A**

  (Sigma type I-A #R4875). 10 $mgml^{-1}$ in autoclaved dH_20 and store at -20°C in 1.2 ml aliquots

Antibody staining

- **10 x DIG Buffer 1**

1 M Tris	500 ml 2 M pH 7.5
1.5M NaCl	87.7 g

  in lL of dH_20. Check final pH is 7.5

- **DIG Buffer 2 (make fresh each day)**

  0.5% (w/v) blocking reagent (Roche # 109 6176) in DIG Buffer 1.

  Dissolve for ~1 hr at 60-70°C

- **DIG Buffer 3 (make fresh each day)**

  1% BSA (Roche 735 086))

  0.3% Triton-X-100 (Sigma T8787)

  Dissolve in DIG Buffer 1

- **DIG Buffer 4 (make fresh each day)**

  1 in 3000 dilution of anti-digoxigenin-AP FAB fragments (Roche #1093-274) in DIG Buffer 3.

- **10 x DIG Buffer 5A**

1 M Tris pH 9.5	250 ml 2 M
1M NaCl	100 ml 5 M

  up to 500 ml with autoclaved distilled H_2O. Check final pH is 9.5

- **10 x DIG Buffer 5B**

  0.5 M $MgCl_2$ in autoclaved distilled H_2O.

- **4-nitro-blue-tetrazolium chloride (NBT) 75 $mgml^{-1}$**

  Roche #1087-479 made up in 50% dimethylformamide (Sigma # D4254), aliquoted and stored in the dark at –20°C.

- **5-bromo-4-chloro-indolylphosphate 4 toluidine salt (BCIP) 50 mgml^{-1}**

  Roche #1383-221. Store at –20°C.

- **Levamisole**

  Sigma #L9756. Make up to 24 mgml^{-1} in distilled H_20 and store at –20°C in aliquots.

- **Calcafluor**

  Sigma $C_{40}H_{42}N_{12}O_{10}S_2Na_2$

- **Entellen neu**

  Merck #1.07961.0500.