PROTOCOL:

WHOLE MOUNT *IN SITU* HYBRIDISATION IN PARASITIC FLATWORMS

(ie. *Hymenolepis microstoma*)

Version 1: 22.04.2009 PD Olson

CREDITS: Based with only slight modification on protocols and methods used by the laboratory of Prof Peter Holland (Zoology, Oxford University, UK) and by other members of Oxford Evolutionary Biology Group. First draft of protocol modified for tapeworms thanks to Pat Dyal (NHM).

GENERAL NOTES:

- Protocol for digoxigenin/alkaline-phosphotase-coupled (DIG-AP) whole-mount *in-situ* hybridization of riboprobes in parasitic flatworms (albeit protocol should have broad applicability and stems from work on animal models ranging from *Xenopus* to polychaetes).
- All steps carried out in 1.5 ml eppendorfs. For larval tapeworm specimens, a flash spin should be performed after each step.
- During wash steps, eppendorfs are placed inside a 50 ml flacon tube which is then place on a horizontal tube roller, providing continuous, gentle agitation.
- The hybridization step is carried out using an agitating heating block which is itself placed on its side on a rocking table, thus providing constant and accurate temperature with constant and gentle agitation. **NOTE** accurate temperate is crucial during the hybridization step as it affects specificity.
- Use filter-tips throughout.
- Pre-warm buffers using a hot water bath on the bench where necessary, allowing the user to pipette the buffer without removing if from the water bath.
- Steps specific to tapeworms are indicated by ❖

Fixation of animals for WMISH

Reagents:

4% Paraformaldehyde in PBS pH 7.5

100% methanol

❖ Mammalian physiological saline (ideally) for mouse dissection (beetle dissections carried out using conditioned water—albeit insect physiological saline would be better).

❖ Adult *Hymenolepis microstoma*:

1. Remove live adult worms from bile duct and anterior small intestine and swirl in fresh saline to remove debris.
2. Individually, transfer worms to near-boiling saline and swirl for ~ 5 seconds to extend and kill worms (ie. 'heat-fix'; **NOTE** live worms placed in paraformaldehyde will contract to 1/5 their full size, obscuring all view of their internal anatomy).

❖ Larval *Hymenolepis microstoma*:

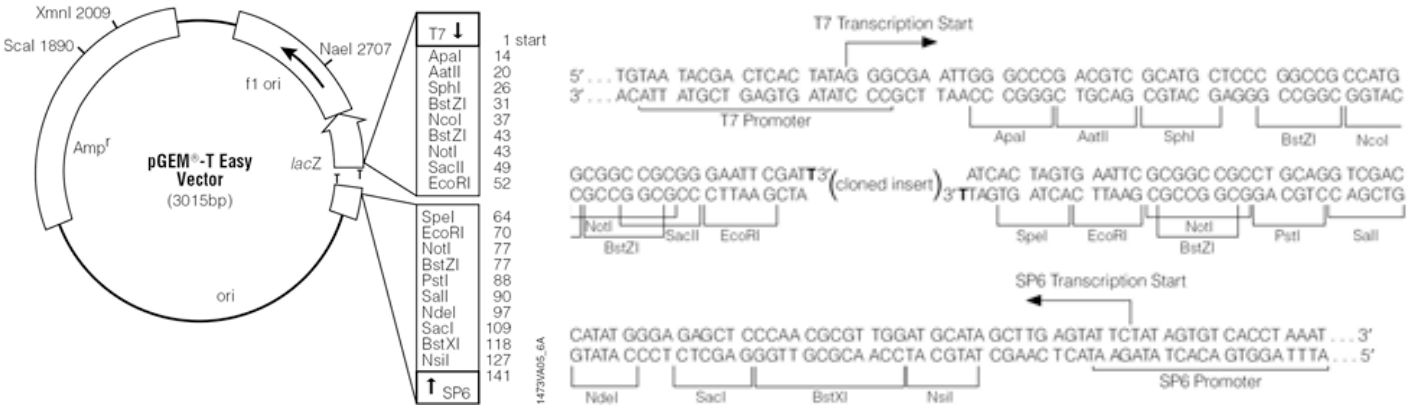
1. Dissect from haemocoel of beetles and transfer to saline or water.
2. Rinse free of debris using fresh saline or water.

3. Place in fresh, refrigerated 4% paraformaldehyde and leave overnight in refrigerator.
4. Replace with 100% methanol and agitate on roller for 10 mins.
5. Exchange methanol and store at -20°C. Specimens can now be stored long term at –20°C or used directly for *in situ* hybridization.

Synthesis of DIG-labeled anti-sense probes

- Anti-sense riboprobes for WMISH should be of sufficient length to produce high-specificity to the target, e.g. 500-2500 bps. Because long probes may be less efficient at diffusing through the tissues, the full length probes can be synthesized and later fragmented using any number of techniques. However, we have thus far not needed to do this using probes ranging in size between 1500-2500 bps on tapeworms.
- After characterizing full or partial mRNA transcripts of interest (via 5' & 3' RACE or EST sequencing), gene specific primers should be designed such that the entire transcript can be amplified in a single piece from a cDNA template.
- The PCR product is cloned using a suitable vector (aka plasmid) with SP6/T7 RNA polymerase promotor sites: e.g. pGEM-T Easy (Promega)
- A number of clones should be sequenced in order to confirm the identity of the product and to determine the orientations of the inserts. The sample should also be quantified using the NanoDrop.
- Based on the insert orientation, either T7 or SP6 RNA polymerase is used to transcribe single-stranded, **anti-sense** "run-off" transcripts (ie. probes) incorporating a digoxigenin-substituted ribonucleotide (ie. DIG-dUTP).
- Many labs transcribe both sense and anti-sense probes for controls (or perhaps to ensure at least one of the probes will be complimentary to the mRNA of interest).
- Prior to DIG-transcription, the vector/insert construct must be linearized by cutting it with an appropriate restriction enzyme (see vector map below).
  NOTE it is essential that the enzyme chosen is downstream of the insert with respect to the polymerase used (otherwise the T7 or SP6 polymerase promoter site will be at the end of the linearized template and the insert sequence will not be transcribed).
  NOTE it is also essential that a single-cutter is used, otherwise the insert will be removed entirely from the vector (and thus from the polymerase promoter site). Use of EcoRI with pGEM-T vectors, for example, will cut on both sides of the insert, thus cleaving the insert from the vector (resulting in separate bands on a gel representing the vector and insert sequences).
  NOTE that after a restriction enzyme has been chosen that satisfies the above criteria, it must also be determined if any restriction sites specific to this enzyme are found in the insert sequence (many bioinformatics programs, such as Sequencher, will provide a restriction map of the input sequence showing all restriction sites present in the sequence). If one or more sites are found then the enzyme can not be used as it will shortened or remove the insert altogether.

Promega pGEM-T Easy vector maps



Plasmid linearization:

1. Do a restriction digest of the vector (aka. plasmid), cutting 5-10 micrograms of DNA.
2. Use 2 μl 10X buffer, 5-10 μl of DNA (ie. vector), 1 μl restriction enzyme, make up vol to 20 μl with ddH2O.
3. Incubate at 37°C for at least 90 mins.
4. Check 1-2 μl of the digest on an agarose gel to ensure that the enzyme has cut to completion (only a single band should appear with the size being the combination of the insert + vector (the pGEM-T Easy vector is 3001 bps). If necessary, continue reaction.
5. Heat inactivate the restriction enzyme at the appropriate temperature (e.g. 65-80°C)
6. Clean using a DNA clean-up kit (eg. Qiagen), elute in 20 μl DEPC-treated ddH2O and store at -20°C.

Synthesis of probe by DIG transcription

Based on Roche DIG RNA labeling with digoxigenin-UTP by in vitro transcription with SP6 and T7 RNA polymerase (*kit #11 175 025 910; or buy individual components: see 'Recipes & Reagents' at end).

1. Add the following to a microcentrifuge tube on ice:

Linearized plasmid DNA	1 microgram
Roche 10X NTP Labeling Mix (*vial 7)	2 μl
Roche 10X Transcription buffer (*vial 8)	2 μl
Roche Protector RNAse inhibitor (*vial 10)	1 μl
RNA Polymerase (SP6 or T7)	2 μl
DEPC ddH2O to a **final volume of 20 μl**	

2. Mix gently and flash spin
3. Incubate for 2 hours at 37°C.
4. Stop reaction by adding 2 of 0.2 M EDTA (pH 8)
5. Aliquot and store at -20°C.

PREPARATION OF DIGOXIGENIN-LABELED PROBES

(via P Dyal)

I. Preparation of template-plasmid DNA

1. Cloned DNA template (PCR product) into appropriate vector for example Bluescript or pGEM that has sites for T7, T3, or SP6 RNA polymerase flanking the multiple cloning site. You should know the orientation of the cDNA insert with respect to the polymerase and restriction sites so that you can determine what polymerase to use to generate an anti–sense run-off transcript (this will be your RNA probe).

2. Prepare plasmid DNA from positive clone. Check orientation of insert and determine appropriate enzyme to use to produce an anti-sense probe **(reverse transcribed in the 3'-5' direction**). Restriction enzymes creating 5'-overhangs should be used; 3' overhangs should be avoided.

3. Estimate concentration of plasmid DNA with Nanodrop or spectrophotometer.

II. Linearisation of template –plasmid DNA

4. Decide on the scale of the reaction (1µg template produces 10 µg of probe). Recommend a restriction digest on 5-10µg of DNA in 20 µl total volume (can lose a lot of DNA during ethanol precipitation).

   - For example Standard restriction digest for *Hymendlepis microstoma* clones, digested with Spe 1. In a sterile tube, assemble in order:

Sterile water	6.8 µl
10 x Restriction buffer	2.0 µl
Aceytlated BSA 10 µg/µl	0.2 µl
Template-plasmid DNA (10 µg)	10.0 µl

Mix by pipetting, then add:

Spe 1 (10U/ µl)	1.0 µl
Final volume	20.0 µl

Mix gently by pipetting, spin briefly.

5. Incubate at 37 ºC for 2-3 hours.

6. Check 1 or 2 µl of the digest on an agarose gel to confirm that the enzyme has cut your plasmid to completion. Recommend that you run approximately the same concentration of uncut plasmid DNA next to the test digest aliquot to do a direct comparison of the cut and uncut DNA.

7. Clean up the digest by doing a phenol/chloroform extraction and an ethanol precipitation.

   - Bring the digest volume to 200 µl

- Add 200 µl (equal volume) of Phenol-chloroform (25:24:1)
- Vortex for 1 min
- Centrifuge at 5000 rpm for 5 min
- Remove the top aqueous phase and transfer to a new tube
- Add 200 µl (equal volume) of Chloroform:isoamylalcohol (24:1)
- Vortex for 1 min
- Centrifuge at 5000 rpm for 5 min
- Remove the top aqueous phase and transfer to a new tube
- Add 20 µl of 3M sodium Acetate pH 5.2 (ie. 0.1vol) and 500 µl of ice cold absolute ethanol (ie. 2.5 vol). Mix by inverting the tube several times
- Place the tube at -20ºC for at least one hour or -80ºC for 30 min
- Centrifuge at max speed (14000 rpm) for 15 min at 4ºC
- Remove the ethanol and wash the DNA pellet with 100 µl of 70% ethanol
- Vortex briefly and Centrifuge @ 14000rpm for 5 min
- Remove as much ethanol as possible and dry the DNA pellet
- Resuspend the DNA in 50-100 µl of DEPC H_2O (depends on size of DNA pellet)
- Check the DNA, by running 1 µl on a 0.8% agarose gel and estimate the DNA concentration at A260 using a Nanodrop or spectrophotometer.
- Store at -20ºC

III. Synthesis of probe - RNA Labelling by in vitro Transcription of DNA with DIG RNA Labelling Mix - Roche: DIG RNA Labeling Kit (SP6/T7) Cat. No. 11 277 073 910

1) ***Perform Transcription Reaction by adding the following to a 1.5 ml microfuge tube on ice***:

   1 µg linearised plasmid
   2 µl 10 x conc. DIG RNA Labelling mix (DIG RNA Labelling kit)
   2 µl 10 x conc. Transcription Buffer (DIG RNA Labelling kit)
   2 µl appropriate RNA Polymerase (SP6, T7, DIG RNA Labelling kit)
   DEPC water to total volume of 20 µl

   Mix components and centrifuge the tube briefly.
   Incubate the tube for 2 hours at 37ºC.

2) Check 2 µl on a 1.5 % agarose gel; run at 200V for 5-10 min.
   RNA at bottom should be approx. 10x stronger than the plasmid template DNA

3) Add 2 µl DNase I, RNase-free to the tube to remove template DNA and incubate for 15 minutes at 37ºC.

4) Add 2 µl of 0.2M EDTA pH8.0 to the tube to stop the polymerase reaction.

5) ***Precipitate RNA Probe - add following to tube:***

   28 µl DEPC water
   5 µl 4M LiCl (0.1 vol)
   150 µl 100% ice cold 100% EtOH (2.5 vol)

- Mix well and pulse spin
- Incubate at -20ºC for 2 hours or -80ºC for 30 min (can leave overnight)
- Centrifuge at 13,000 x g for 30 min at 4ºC
- Decant the ethanol and wash the RNA pellet with 200 µl of ice cold 70% ethanol
- Centrifuge at 13,000 x g for 5 min at 4ºC
- Decant the ethanol and dry the pellet briefly under vacuum.
- Redissolve the RNA probe in 50 µl of DEPC treated water.
- Check 2 µl on a 1.5 % agarose gel; run at 200V for 5-10 min
- Estimate the concentration of the probe using the Nanodrop or spectrophotometer.
  Note: For RNA $A260_{nm}$ of 1 = ~ 40 µg/ml. Store aliquots at -80ºC.

Whole-Mount In Situ Hybridization

DAY 1 (~7 hours):

Reagents

100% Ethanol

75% Ethanol/PBSAT

50% Ethanol/PBSAT

PBSAT (1xPBS+0.1% Tween-20)

Thaw Proteinase K 25 mg/ml (50-100 µl)

0.1M Triethanolamine (TEA) pH 7-8 (~100-200 ml)

Acetic Anhydride (125-250 µl)

4% paraformaldehyde in PBS pH 7.5

Hybridization Buffer (-)

Hybridization Buffer (+)

Rehydration of specimens:

Allow the specimens to warm to RT before proceeding

Rehydrate by washing in:

1. **95% EtOH**, 5 min on a roller
2. Wash **once** in **75% EtOH/PBSAT**, **10 min** on roller.
3. Wash **once** in **50% EtOH/PBSAT**, **10 min** on roller.
4. Wash **three times** in **PBSAT** for **5 min** on roller.

Proteinase K treatment and re-fixing

5. Thaw an aliquot of the stock Proteinase K (14-22 mg/ml; -20ºC). Keep on ice **no longer than 10 – 15 min** until adding to specimens.
6. Add 1.5 ul of Proteinase K (14-22 mg/ml) to each vial containing **2 mls** of PBSAT.
7. Incubate at room temperature for **10-20 min**. Do not agitate!
8. Rinse **twice** in **0.1 M Triethanolamine** (TEA), pH 7.8, for **5 min**; 5 mls per vial.
9. Add **12.5 µl acetic anhydride** to the second wash (fume hood). Keep in the fume hood and swirl often as the anhydride mixes badly. **After 5 min** add another **12.5 µl acetic anhydride** for **5 min.**
10. Wash **twice for 5 min** with PBSAT on roller.
11. Re-fix for 20 min, in **4% paraformaldehyde in PBSAT**, on roller.

NOTES

Rehydration:
Rehydration into PBSAT from ethanol is done gradually to prevent the formation of bubbles in specimens. PBSAT is used because the detergent (Tween) prevents the specimens from sticking to each other. Furthermore, phosphate buffered saline solution helps to neutralize the negative charge of mRNA so that the probe can bind better to it.

Proteinase-K Treatment:
The purpose of this step is to permeabilize the specimen/tissue by removing some of the outer proteins which allows better probe penetration for deeper tissues. This step can be omitted if the staining is restricted to superficial tissues. Proteinase K treatment requires careful monitoring and should not be prolonged as it will degrade the architecture of the tissues.

Trethanolamine:
The triethanolamine is a buffer without free amines which is necessary for the next step.

Acetic anhydride:
Acetic anhydride acetylates free amines to neutralize positive charge so that the probe will bind specifically to mRNA rather than nonspecifically due to electrostatic interactions. The acetic anhydride is sensitive to water vapor in the air, so be sure to pipette it only right before you will be using it. Keep lid on acetic anhydride closed at all times.

Re-fixing:
Refix the specimen/tissue to insure specimen/tissue integrity after the PK step.

Hybridization:
Note: for the following pre-hybridization and hybridization steps, total yeast RNA must be in the hybridization buffer; whereas you don't need it in the hybridization buffer used to equilibrate the specimens.

12**.** Wash **five times for 5 min** or longer in PBSAT, to wash off excess paraformaldehyde.

Equilibration of specimens/tissues in hybridization buffer

13. Add **250µl of hybridization buffer (-) to 1 ml PBSAT** and allow specimens to settle.
14. Remove the buffer and replace with **1 ml of hybridization buffer (-).** Transfer carefully to 1.5 ml tubes.
15. Incubate @ 60°C for 10 min (use the shaking block).

Prehybridization

16. Replace the **hybridization buffer (+)** with 1 ml of fresh hybridization buffer (+) (warmed to 60°C).
17. Prehybridize for 2 hours in incubator, tube on its side @ 60°C using the "patented" horizontal tube rocker. ***Note: Best to leave overnight or for @ least 5-6 hours***.

DAY 2-Hybridization of Probes

Reagents

Hybridization (+) buffer

1µg/ml of digoxigenin–labeled riboprobe

Hybridization

18. Prepare RNA probe: Denature probe (1µg/ml) at 80°C for 3 min, then add to **1 ml of pre-warmed hybridization buffer (+)** @ 60°C. Generally, we use 2-4 ul of DIG prep per ml of hybridization buffer.
19. Remove prehybridisation solution and replace with **1 ml of pre-warmed hybridization buffer (+)** containing **1µg/ml of digoxigenin–labeled riboprobe**.
20. Hybridize @ **60°C overnight** (24 hours).

DAY 3-Antibody Hybridization

Reagents

Hybridisation (+) buffer

2 x SSC + 0.1% Tween-20

0.2 x SSC + 0.1% Tween-20

MAB (100mM Maleic Acid, 150mM NaCL, pH 7.8, 0.1% Tween-20)

} **Pre-warm all wash solutions to 60°C**

MAB + 2% (w/v) Blocking Reagent (BMB) + 20% (v/v) Lamb serum

Sheep Anti-DIG-AP FAB fragments (ie. alkaline-phosphotase-coupled antibody)

Probe removal and washing

21. Carefully remove probe from the vial. Store @ - 80°C in screw-cap microtube. Probe can be reused several times. N.B. probes can actually work better with repeated use as the background staining can be reduced in subsequent rounds of *in situ*.
22. Rinse in **2 ml of pre-warmed hybridization (+) buffer**, wash **@ 60°C for 10 min, twice**. ***Note: While changing solutions have tubes on a hot block to keep specimens @ 60°C at all times!***
23. Wash **three times** in **2 x SSC + 0.1% Tween-20 (warmed to 60°C) for 20 min.**
24. Wash **three times** in **0.2 x SSC + 0.1% Tween-20**, **@ 60°C for 30 min.**
25. Wash **twice** in **Maleic acid buffer (MAB)** for **15 mins @ RT** on roller**.**

Antibody incubation and washing

26. Pre-incubate in **2ml MAB + 2% BMB + 20% heat treated lamb serum** and **rock for 2 hours @ RT.**
27. Remove solution and replace with fresh solution containing **1/2000 dilution of affinity purified sheep anti digoxygenin antibody coupled to AP**. Place small tubes in 50 ml Falcon tubes with support on **rocker overnight at 4°C**.

NOTES

BMB is a blocking reagent to prevent non-specific antibody binding

DAY 4-Colour Development

Reagents

MAB
Alkaline Phosphatase Buffer (make up fresh each time)
NBT/BCIP
PBSAT
4% Paraformaldehyde in PBS pH 7.5
50% EtOH/PBS
70% EtOH/PBS
90% EtOH/PBS
100% EtOH
BA:BB clearing agent (1:1 Benzyl Alcohol/Benzyl Benzoate)

Washing

28. Remove antibody and keep at 5ºC for future use.
29. Wash **three times for 5 min** with **MAB**, mixing gently.
30. Transfer to large vials again and wash **three times for 1 hour (3 x 1 hour washes)** using aspirator, rocking during the washes

31. Wash **once for 3 min** in **Alkaline Phosphatase buffer** @ RT.
32. Wash **once for 10 min** in **Alkaline Phosphatase buffer** @ RT.

NOTES

Alkaline Phosphatase buffer:
This buffer inhibits endogenous phosphatases

Washing in PBSAT:
The color reaction is stopped by a change in pH.

Post- fixation:
The paraformaldehyde also stops the chromogenic reaction as well as stabilizing the stain in the specimens.

Chromogenic Colour Reaction

33. Add **5 µl NBT/BCIP** staining mix and place in the dark until colour develops, which may be as little as minutes or as long as days. In our experience, full colour development in adult worms has taken ~24 hours or longer (whereas larvae stain more quickly). Leave specimens in the refridgerator.
34. After staining wash **twice** in **PBSAT** for 15 min, to stop the staining reaction.
35. Specimens can be post-fixed in **4% paraformaldehyde in PBS** for 1 hour at RT or overnight at 4ºC. This increases the rigidity of the specimens, stops and stabilizes the colour reaction, but is not necessary in our experience.

Clearing

36. Gradually dehydrate using an ethanol dilution series.

- **50% EtOH/PBS** for **5 min** on roller at RT.
- **70% EtOH/PBS** for **5 min** on roller at RT
- **90% EtOH/PBS** for **5 min** on roller at RT
- **100% EtOH** for **5 min** on roller at RT

37. Clear dehydrated specimens in a 1:1 solution of Benzyl Alcohol:Benzyl Benzoate (N.B. will melt plastic, such as NUNC multi-well trays!)
38. Specimens are now ready to be imaged. Store specimens in the refridgerator in clearing solution. In our experience, colour will remain for weeks or months.

RECIPES & REAGENTS (w/ stock no.s)

5X Transcription buffer (Promega P118B)

10X DIG NTP mix (Roche 130 377 20)

Acetic anhydride (Sigma A6404)

Anti-Digoxigenin-AP Fab fragments (Roche 11 093 274 910)

BMB (Roche 11 096 176 001)

CHAPS (Sigma C3023)

DEPC ddH2O (Sigma D5758): 0.1-0.5% in ddH2O, autoclave

Denhardt's 50X stock (Sigma D2532)

DTT (Promega P117B)

Formamide (Sigma F7508)

Heat-treated Lamb Serum (Gibco/Invitrogen 16070-096)

Heparin (porcine sodium salt) (Sigma H3393)

Hybridization buffer (100 mls):

50% formamide	50 ml
5X SSC	25 ml of 20X
1 mg/ml total yeast RNA	2 ml of 50 mg/ml
100 ug/ml heparin	100 ul of 100 mg/ml
1x Denhart's	2 ml 50X
0.1% Tween 20	
0.1% CHAPS	
10 mM EDTA	2 ml 0.5M
DEPC water	

MAB (Maleic acid buffer) (Sigma M0375)

PBS (Phosphate-buffered saline) tabs (Oxoid BR014G, 100 tabs)

PBSAT (Phosphate-buffered saline with TWEEN at 0.1%) N.B. also known as 'PBT'

Proteinase K (Roche 03115 828 001)

NBT/BCIP substrate (Roche 11 681 451 001)

RNAsin (50 units) (Promega N261A)

SP6 & T7 RNA polymerases (Ambion T7: 2716; SP6: 2702)

SSC 20X = 175g NaCl + 88.2g TriNaCitrate in 1L ddH2O

TEA (Triethanolamine) 0.1M pH 7.8 (Sigma T-1377)

Total Yeast RNA (Roche 10 109 223 001)

Tween 20 (Sigma P9416)

Receipes for reagents used in *in-situ* hybridisation

Reagents needed:

DEPC Treated ddH_2O

1 x PBS pH 7.5

Dissolve 10 Tablets into 1 litre of dd H_2O,
DEPC treat- add 1ml of DEPC to 1 liter of PBS, let it sit overnight in a fume hood and.
Autoclave

PBSAT (1 x PBS + 0.1% Tween-20)

Dissolve 10 Tablets into 800 ml of dd H_2O,
Adjust to 1 liter with dd H_2O
Add 1ml of DEPC into solution. Shake well and leave overnight at 37ºC with the bottle cap-loose
Sterlize by autoclaving

When cool add 1 ml of Tween 20
Filter-sterilize, and store @ RT

75% EtOH in PBSAT

375 ml EtOH + 125 ml PBSAT
Store @ RT

50% EtOH in PBSAT

250 ml EtOH + 250 ml PBSAT
Store @ RT

Proteinase K @ 25 mg/ml

Dissolve 0.125 g of Proteinase K in 5 ml of Sterile DEPC H_2O.
Aliquot into 1 ml Eppendorfs and store @ -20˚C

0.1 M Triethanolamine (TEA), pH 7 – 8

Stock Triethanolamine (T-1377; Sigma) – **Concentration 7.53M**

Prepare a 1/75.3 dilution of the stock TEA:-

6.65 ml of 7.53M TEA stock
493.35 ml of autoclaved DEPC treated H_2O

Note: use pH strips to adjust pH with HCL

Filter-sterilize, and keep @ RT

4% (w/v) Paraformaldehyde in PBSAT

Dissolve 8g Paraformaldehyde in 200 ml PBSAT

Filter-sterilize (0.2 μ filter units) and store @ - 20°C in 50 ml aliquots

10% CHAPS (w/v solution)

Dissolve 5 g of CHAPS in 40 ml of sterile DEPC ddH_2O.

Adjust volume to 50 ml with sterile DEPC ddH_2O. Store in aliquots @ -20C.

10% Tween 20 (v/v solution)

10 ml of Tween 20

90 ml of sterile DEPC treated ddH_2O

Heparin (100mg/ml) Stock

Dissolve 0.5 g of Heparin in 5 ml of sterile water (not DEPC water).

Store in 0.15 ml aliquots @ -20ºC.

Yeast RNA (100 mg/ml) Stock

Store in 0.15 ml aliquots @ -20ºC

0.5M EDTA, pH8.0

From SIGMA, cat# E7899; DNase, RNase free

8 M Lithium Chloride

From SIGMA, cat# L7026; DNase, RNase free

20 x SSC (3M NaCl, 0.3 M tri-sodium citrate), pH 7.

175.3 g NaCl
88.2 g Tri-sodium citrate
DEPC-ddH_2O to ~ 800 ml

Dissolve solutes. Adjust pH to 7.0 with HCl.

Adjust volume to 1 liter with DEPC-ddH_2O. Sterilize by autoclaving

HYBRIDISATION BUFFER

FINAL CONCENTRATION	**Stock**	**100 ml**
50% Formamide (deionized)	100 %	50.0 ml
5 x SSC (pH7)	20 x SSC	25.0 ml
1mg/ml total yeast RNA	100 mg/ml	1.0 ml
100 ug /ml Heparin	100 mg/ml	0.1 ml
1 x Denhardt's	50 x Denhart's	2.0 ml
0.1% Tween 20	10% Tween 20	1.0 ml
0.1% CHAPS	10% CHAPS	1.0 ml
10 mM EDTA pH 8.0	0.5M EDTA pH 8.0	2.0 ml
DEPC water		17.9 ml

Note:

- **Make up Hybridisation buffer without the yeast RNA. [Hybridisation (-) buffer].**
- **Aliquot 9.9 ml of the buffer into sterile 15 ml Falcon tubes and freeze @ -20˚C.**
- **To use thaw an aliquot of the hybridisation buffer and add 100 µl of 100mg/ml total yeast RNA. [Hybridisation (+) buffer].**

Formamide and Hybridization:
Formamide lowers the melting point of nucleic acids so that the strands separate more readily. DNA is normally more stable in a double-stranded structure (even if every base isn't complementary) and less stable when single-stranded, so formamide must increase the stability of single-strandedness. In *in situ* hybridization, an RNA probe binds to mRNA that is already single-stranded. mRNA does not gain any stability by being a hybrid unless the probe is specific and can bind properly, thus increasing stability. For example, in the presence of formamide, a U nucleotide would rather bind to an A than nothing (binding to specific probe is better than staying single stranded), but a U nucleotide would rather bind to nothing than a G (binding to non specific probe is worse than binding to nothing)
Denhardt's solution: **A solution commonly used during probe hybridisations. Denhardt's solution is a mixture of high-molecular weight polymers capable of saturating non-specific binding sites and artificially increasing the concentration of available probe. It is prepared as a 50X solution with the following composition: 1% Ficoll (type 400), 1% polyvinylpyrolidone, and 1% bovine serum albumin. NOTE: For use in RNA work, Dissolve components in DEPC treated or sterile ddH2O.**
Tween® 20
Tween 20 is a polysorbate surfactant whose stability and relative non-toxicity allows it to be used as a detergent and emulsifier or as a blocking agent. Tween 20 is also needed in the buffer to further prevent the non-specific binding.

2 x SSC+ 0.1 % Tween 20

100 ml 20 x SSC
890 ml sterile ddH_2O
10 ml 10% Tween 20

Filter-sterilize and store @ RT

0.2 x SSC + 0.1 % Tween 20

10 ml 20 x SSC
980 ml sterile ddH_2O
10 ml 10% Tween 20

Filter-sterilize and store @ RT

1M Maleic Acid Buffer, pH 7.8 – 1 Liter

Dissolve 116.1 g Maleic acid in a small amount of DEPC dd H_2O (300 ml)
pH to 7.8 with lots of 10N NaOH
Sterilise by autoclaving

MAB (Maleic Acid Buffer), pH 7.8 – 1 Liter

1) MAB made up from stock solutions:

	Final Concentration
100 ml 1M Maleic acid	0.1 M
30 ml 5M NaCl	0.15 M
10 ml 10% Tween 20	0.1% (v/v) Tween 20
860 ml Sterile DEPC dd H_2O	

2) MAB made up from stock chemicals:

100 mM Maleic acid	23.21 g
150 mM NaCl	17.53 g
0.1% Tween 20	20 ml of 10% Tween 20 stock

Dissolve in approximately 1800 ml dd H_2O, adjust pH to 7.5 with 10 N NaOH, volume to 2 L, and store @ RT

MAB + 2% (w/v) Blocking Reagent

10 g Blocking Reagent in 500 ml MAB
Autoclave and store @ 4°C

Lamb Serum

Lamb serum purchased from Gibco BRL (cat # 16070-096) is thawed @ RT.
Heat-inactivate complement @ 60°C for 30 min to destroy endogenous alkaline phosphatase activity.
Centrifuge @ 10,000 rpm for 20 min @ 4°C to remove particulate material
Store @ - 20°C in 25 ml aliquots

MAB + 2% (w/v) Blocking Reagent + 20% (v/v) Lamb serum

20 ml of heat-inactivated Lamb serum + 80 ml MAB with 2% Blocking Reagent
Make fresh as needed, and do not store more than 1 – 2 days @ 4°C

Anti-Digoxigenin AP, Fab (Antibody)

From Roche, cat # 11 093 274 910 (150 U in 200 μl)

1M Tris-HCl, pH 9.5

Dissolve 121.14 g Tris (hydroxymethyl) aminomethane, (Tris, MW = 121.14) in 800 ml dd H_2O
Adjust pH to desired value by adding concentrated HCl:

- pH 9.5 : ~ 8 ml

Adjust volume to 1 liter with dd H_2O
Sterilize by autoclaving and store @RT. **Do not treat Tris solutions with DEPC.**

1 M $MgCl_2$ - 1 liter

Dissolve 203.31 g magnesium chloride-6 H_2O, (MW=203.31) in 800 ml dd H_2O
Adjust volume to 1 liter with dd H_2O
Sterilize by autoclaving and store @RT

5M NaCl – 1 liter

Dissolve 292.2 g sodium chloride, (MW=58.44) in 800 ml dd H_2O
Adjust volume to 1 liter with dd H_2O
Sterilize by autoclaving and store @RT

Alkaline Phosphatase Buffer

Final Concentration	**50 ml**	**100 ml**
100 mM Tris Cl, pH 9.5	5.0 ml 1 M Tris Cl, pH 9.5	10.0 ml of the same
50 mM $MgCl_2$	2.5 ml 1 M $MgCl_2$	5.0 ml of the same
100 mM NaCl	1.0 ml 5 M NaCl	2.0 ml of the same
0.1% Tween 20	0.5 ml 10% Tween 20	1.0 ml of the same
Sterile ddH_2O	41.0 ml	82.0 ml

Store @ 4°

NBT/BCIP Stock Solution

From Roche, 11 681 451 001

Solution of 18.75 mg/ml nitroblue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt in 67% (DMSO) (v/v)

50% EtOH/PBS

125 ml EtOH + 125 ml PBS
Store @ RT

70% EtOH/PBS

175 ml EtOH + 75 ml PBS
Store @ RT

90% EtOH/PBS

225 ml EtOH + 25 ml PBS
Store @ RT

100% EtOH

BA:BB clearing agent (1:2 Benzyl Alcohol/Benzyl Benzoate)

100 ml Benzyl alcohol

200 ml Benzyl Benzoate

Store @ RT

ORDERING INFORMATION (UK)

Item Description	Company	Catlogue Number	Pack Size	Cost per Pack- (£)
DEPC (Diethyl pyrocarbonate)	SIGMA	D5758	25 ml	44.50
Triethanolamine (TEA)	SIGMA	T-1377	500 ml	14.50
Acetic Anhydride	SIGMA	A6404	500 ml	11.50
Sodium citrate Tri-Basic	SIGMA	S4641	500 g	15.60
Maleic Acid	SIGMA	M0375	500 g	13.50
CHAPS	SIGMA	C3023	5 g	78.25
Tween-20	SIGMA	P9416	100 ml	21.30
Deionized Formamide	SIGMA	F9037	100 ml	26.90
50 x Denhardt's Solution	SIGMA	D2532	5 ml	43.60
Heparin sodium salt	SIGMA	H1027	50,000 U	21.30
H_2O	SIGMA	W4502	1-Liter	35.60
RNaseZAP®	SIGMA	R2020	250 ml	39.80
0.5 M EDTA pH8.0	SIGMA	E7899	100 ml	22.90
8 M Lithium Chloride	SIGMA	L7026	100 ml	24.00
PBS- tablets	OXIOD	BR0014	100-Tablets	5.39
Total Yeast RNA	ROCHE	10 109 223 001	100 g	35.80
Blocking Reagent (BMB)	ROCHE	11 096 176 001	50 g	59.80
Anti-Digoxigenin-AP, Fab fragments (from sheep)	ROCHE	11 093 274 910	150 U (200 µl)	147.20
NBT/BCIP Stock Solution	ROCHE	11 681 451 001	8 ml	57.80
DIG RNA Labeling Kit (SP6/T7)	ROCHE	11 277 073 910	40 µl (20 reactions)	117.20
Lamb Serum	Invitrogen	160 70 096	500 ml	42.64
Benzyl Alcohol 99^{+}% pure	FISHER	10584 5000	500 ml	24.27
Benzyl benzoate 99^{+}% pure	FISHER	10586-2500	250 ml	12.33