# WHOLE MOUNT *IN SITU* HYBRIDIZATION OF MOUSE EMBRYOS

(June, 1997 - optimized by N. Kertesz, L.Leyns & E. De Robertis)

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## Preparation of embryos

Dissect embryos in cold PBS, change solution often.

Punch a hole in brain cavities for embryos older than 9 dpc.

Transfer after dissecting a few embryos to a 5 ml screw cap flat bottomed glass vial containing 4% paraformaldehyde (freshly made. Add powder paraformaldehyde to PBS and heat to 60 °C with stirring until clear) Store on ice.

When all the embryos of the same mother are dissected, renew the 4% paraformaldehyde and incubate at 4 °C for 4 hrs for 7.5d embryos or overnight for older embryos (or overday if dissection is done in the morning).

The next day, wash 2x with PBSw (PBSw=PBS with 0.1% Tween-20)

Dehydrate with methanol series (25%, 50%, 75%, 100% in PBSw). Change 2x in 100% methanol.

Store the embryos at -20 °C (up to 2 months).

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## Preparation of the probe

Probes are prepared as Digoxigenin labeled RNA. The labeling mix as well as all antibodies are purchased from Boehringer

All conditions and solutions should be totally RNAse free.

Use gloves and aerosol barrier tips.

Linearize the plasmid and check the digest.

Phenol extract.

Extract twice with chloroform:isoamyl alcohol (24:1)

Ethanol precipitate (1/2 vol 7.5 M NH4 OAc + 2.5 vol 100% ethanol. Rinse with 125µl 75% ethanol. Let dry with caps open for 10 minutes. )

Resuspend in suitable volume of nuclease free water.

Measure concentration.

Set up transcription reaction

200ng DNA

2 µl 10X transcription buffer (Stratagene)

2 µl labelling mix

1 µl RNAGUARD (Pharmacia)

1 µl RNA polymerase (SP6, T3 or T7)

Add water to 20 µl

Incubate for 2 hours at 37 °C.

Run on 1% agarose gel (1.5µl probe + 5µl of 1.2X running buffer.) for a short time. The RNA should appear as a single band with little degradation product and about 10 times more intense than the DNA band.

Remove unincorporated free nucleotides with Quick-Spin Columns.

Remove the caps (top first not to create air bubble trapped in the column) and spin for 5 min, @ 4 °C, 1800 rpm in the Sorval swing bucket centrifuge.

Remove the eluate and centrifuge 5 min again.

Put the column in new tubes, add the transcription reaction onto them and spin 15 min.

The volume of the final eluate should be around 30-40µl .

Run a gel (loading 1.5µl in 5µl loading buffer) to quantify the yield and to determine the amount to be used for the *in situ*.

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**General Comments**

Don't let the embryos dry at any stage as the amount of background will increase. It is prefered to leave the embryos in a small volume of the solution and to add the next solution to it.

Treat all solutions with DEPC (add 0.1% DEPC, incubate with agitation overnight and autoclave 40 minutes) (for Tris solution, use DEPC treated water, do not treat the solution).

Filter all solutions (to remove particles that will stick to the embryos).

Rinse the hybridization vials and caps with RNAZap and rinse at least 5 times with DEPC water.

Make all the fixations, rinses, washes until the pre-hybridization step on ice except the proteinaseK treatment.

Use gloves and aerosol barrier tips for changing the solutions from the fixation step to the end of hybridization.

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## In Situ hybridization

### Day 1

Prepare fresh 4% paraformaldehyde - 0.2% Glutaraldehyde in PBS. About 5 ml will be needed for each sample after proteinaseK treatment.

Prepare hybridization solution. For 50 ml of hybridization solution dissolve;

Hybridization mix recipe (50 ml):

0.5 g Boehringer Block

25 ml formamide

12.5 ml 20X SSC, pH 7

Heat to 65 °C for about 1 hr. Once dissolved add:

6 ml $H_2O$

5ml 10mg/ml torula RNA(heat 2 min at 65 C to clear)

100 µl 50mg/ml heparin

250 µl 20% Tween-20

500 µl 10% CHAPS

500 µl 0.5 M EDTA

Filter the solution. The hybridization solution can be prepared before, aliquoted and stored at -20 °C.

Rehydrate the embryos through 75, 50 and 25% methanol series in PBSw. Incubate each step for 5 min. on ice.

Wash 3 times for 5 min. with PBSw on ice.

Change to 1ml of **4.5** µg/ml Proteinase K in PBSw.
Incubate for **3** min for 6dpc at RT, 5 min for 7.5dpc, 7 min for 8.5 dpc, 9 min for 9.5 dpc, 11 min for 10.5 dpc, 13 min for 11.5 dpc.
Staining for highly expressed gene requires less digestion, but for low expression genes longer digestion may help to get stronger staining. Make sure to thaw the proteinase K stock completely and vortex to dissolve precipitate at the bottom of the tube.
Use aliquots of the proteinase K stock 10mg/ml, do not thaw-freeze repeatedly. (Incubation times have to be optimized for each stock.)

**Stop digestion by washing in freshly prepared 2mg/ml glycine in PBSW**

Rinse in PBSw.

Wash 2 times with PBSw for 5 min .

Refix in 5 ml of 4% paraformaldehyde-0.2% glutaraldehyde in PBSw for 15 min.

Rinse in PBSw.

Wash **3** times with PBSw for 5 min. each.

Wash in 1 ml of 50% PBSw:50% hybridization solution, followed by 100% hybridization solution for about 3 min. each standing.

Replace 900 µl of fresh hybridization mix in each glass vial.

**Prehybridize samples for 3 hrs at 65 °C.**

Heat 200 ng of the RNA probe in 100 µl of hybridization mix to 95 °C for 5 min.

Add the probe/hybridization mix to the embryos. The final probe concentration should be about **200ng/ml.**

**Hybridize overnight at 70 °C in a water bath.**

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## In Situ hybridization

## DAY 2

Remove hybridization solution and add 800µl of prehybridization solution. Wash for 5 minutes at 70 °C.

Add 400µl of 2X SSC, ph 4.5 (without removing prehybridization solution.) C. Repeat the addition of the 2XSSC wash twice more.

Remove the mix and wash twice, 30 min each time, in 2XSSC pH7/0.1% CHAPS 70 °C.

Wash twice, 10 min each, in Maleic Acid Buffer ( MAB; 100 mM maleic acid, 150 mM NaCl; pH 7.5) at room temperature. Wash twice, 30 min each time, in MAB at 70 °C.

Wash twice 10 min each in PBS at room temperature.

Wash 5 min in PBSw at room temperature.

Incubate the embryos in 1 ml antibody buffer for at least 2 hours at 4 °C with rocking.

BMblock-mouse antibody buffer 2.5ml needed for each sample:

10% Goat serum (heat inactivated 30 min at 56 °C)

1% boehringer blocking reagent in PBSw

Heat the mixture at 65 °C until total dissolution, filter through 4.5 micron filters (several may be needed), then cool on ice.

During the blocking step, preabsorbe the antibodies. The dilution for the Alkaline phosphatase conjugate (AP) is 1/10000 from a stock of 150 units/200 µl (Boehringer). Dilute the antibody in 1.5 ml of antibody buffer and incubate rocking for at least 2 hours at 4 °C. Use this solution to replace the blocking solution.

Replace buffer with diluted antibody and incubate overnight at 4 °C rocking.

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## In Situ hybridization

### DAY 3

Fast wash embryos with 0.1% BSA in PBSw.

Do another 5 washes with 5 mls 0.1%BSA in PBSw (fill to the top to minimize air bubble) rotating for 45 min. each.

Wash twice, 30 min. each in PBSw.

Take out staining solutions to warm in RT.

Wash the embryos in AP1 buffer (100 mM Tris 9.5; 100 mM NaCl; 50 mM MgCl2) rocking for 10 min, two times each, at RT.

Replace with 1 ml BM purple and rock slowly in the dark. BM purple staining takes a few hours to several days, if necessary leave at 4 °C overnight or until background appears.

Stop staining reaction by washing in at least three changes of PBS.

After staining, dehydrate through methanol series (25%, 50%, 75%, 2x 100%) and store in methanol at -20 °C.

Take pictures after placing back in methanol. BM purple becomes more blue and intense in methanol.

# Anderson Lab In Situ Hybridization Protocols

## March 1995

These protocols describe non-radioactive methods for *in situ* hybridization on frozen sections, whole mount embryos and on cultured cells. They have been freely adapted and modified to greater or lesser extents from the protocols of Richard Harland, David Wilkinson, Domingos Henrique, Andy McMahon, Tony Campagnioni, and others. We wish to thank Eric Mercer, Lukas Sommer, Lisa Banner, Joe Verdi, Susan Birren and especially Li-Ching Lo for their efforts in designing and troubleshooting the protocols. The protocol for *in situs* on cultured cells is still being optimised, but the current version is the best we have working so far. Any queries, comments or suggestions should be directed to Andy Groves at grovesa@starbase1.caltech.edu

Good Luck!

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## I: In Situ Hybridization of Frozen Sections

Sections are collected on RNAse - free slides coated with TESTA, dried in air for two hours and then stored at -20deg.C. It is sometimes necessary to wash the slides in DEPC-PBS and three changes of DEPC-water before storing at -20deg.C. This depends on both the probe and embedding material used. Details of how to prepare RNAse - free slides are in the Appendix.

### A: Pre-Treatment of Sections

Warm slides to room temperature and dry at 50deg.C for 15 minutes.

Fix in 4% paraformaldehyde in DEPC-PBS at room temperature for 20 minutes.

Wash twice in DEPC-PBS at room temperature for 5 minutes.

Treat slides with 50ug/ml Proteinase K in PK buffer at room temperature for between 8-15 minutes depending on the age of the embryo.

Wash once in DEPC-PBS at room temperature for five minutes. Fix in 4% paraformaldehyde in DEPC-PBS for 15 minutes. Rinse once in DEPC-water.

Place slides in an RNAse-free glass trough with a stir bar. Add 250ml 0.1M RNAse-free triethanolamine-HCl pH 8.0. Add 0.625ml acetic anhydride **(CARE!)** with constant stirring. Turn off stirrer when the acetic anhydride is dispersed and leave for a further 10 minutes.

Wash slides in DEPC-PBS at room temperature for five minutes.

Prehybridise for 3-4 hours at 60deg.C. Replace with 1-2ug/ml of probe and continue incubation for a further 12-16 hours.

**N.B.**We have found that the most effective way to carry out the hybridisation is in slide mailers. It is a good idea to thoroughly seal the lids of the slide mailers with parafilm to prevent evaporation of probe.

### B: Washing Steps

**N.B.** After hybridization, it is not necessary to use RNAse-free buffers.

Place slides in a trough with a stir bar. Wash in 1xSSC at 60deg.C for 10 minutes.

Wash in 1.5xSSC at 60deg.C for 10 minutes. Cool slides to 37deg.C.

Wash twice in 2xSSC at 37deg.C for twenty minutes each.

Treat with 0.2ug/ml RNAse A in 2xSSC at 37deg.C for 30 minutes.

Wash in 2xSSC at room temperature for 10 minutes.

Wash twice in 0.2xSSC at 60deg.C for 30 minutes each.

Wash twice in PTw at 60deg.C for 10 minutes each.

Wash in PTw for 10 minutes at room temperature, followed by PBT for 15 minutes at room temperature.

Incubate slides in 20% heat-inactivated sheep serum in PBT for between one and five hours at room temperature. For some probes, the longer incubation seems to cut down on background.

#### C: Antibody Visualisation of Digoxygenin

Incubate slides with pre-absorbed anti-digoxygenin antibody (coupled to alkaline phosphatase) diluted to a final concentration of 1:2000 in 20% sheep serum in PBT at 4deg.C overnight.

Wash three times in PBT at room temperature for 30 minutes each.

Wash twice in Alkaline Phosphatase buffer (first wash without levamisole, second wash with) at room temperature for 5 minutes each.

For every ml of Alkaline Phosphatase buffer, add 4.5ul of NBT and 3.5ul of BCIP, and develop in the dark for between 2-20 hours, depending on the abundance of the RNA. Wash slides with PBS. Since the alkaline phosphatase enzyme is very stable, it is possible to wash out the NBT/BCIP, replace with alkaline phosphatase buffer , and to continue the reaction at a later time.

Fix slides in MEMFA for at least 15 minutes at room temperature. Mount slides.

### II: Whole Mount In Situ Hybridization.

Embryos should be dissected free of any extra-embryonic membranes, fixed in 4% paraformaldehyde in DEPC-PBS for 2 hours at room temperature (or 4deg.C overnight), washed in DEPC-PBS, and then stored in 100% methanol at -20deg.C until required. It may be necessary to partially dissect the embryos (especially the hindbrain region) or puncture them with a fine needle to allow free exchange of reagents.

In general, we do the washes and incubations in 15 ml tubes containing about 5-6 ml liquid. The tubes are rocked gently on a rotating platform to allow thorough exchange of solutions.

**A: Pre-Treatment of Embryos**

Rehydrate the embryos in:
75% MeOH : 25% PTw 5 minutes at room temperature
50% MeOH : 50% PTw
25% MeOH : 75% PTw
100% PTw

Wash twice in PTw for 5 minutes each.

Treat embryos with 10ug/ml proteinase K in PTw for 15-60 minutes. This is a critical step, as over-digestion will destroy the embryos, and under-digestion will give a poor signal. Exact times should be determined for each embryo species and age. Treat the embryos very gently after this step until they are re-fixed.

Rinse once gently in PTw. Re-fix embryos in 4% paraformaldehyde + 0.1% glutaraldehyde for 20 minutes at room temperature.

Wash twice in PTw for five minutes each at room temperature.

Transfer embryos to a 2ml Eppendorf tube. Remove as much liquid as possible, taking care to avoid damaging the embryos. It's better to remove too little than too much. Replace with 1 ml hybridisation mix. Remove this mixture and replace with fresh hybridisation mix.

Pre-hybridise the embryos at 70 deg.C for between 1 and 3 hours. Add probe to a final concentration of about 1ug/ml, and hybridise overnight at 70deg.C.>

(N.B. The subsequent washes with hybridisation mix can be quite costly. The Appendix contains an alternative hyb recipe that uses much less tRNA and works fine for whole mounts. I've tried it a couple of times on sections and it seems to work OK too, but proceed with caution.)

**B: Washing Steps**

Rinse twice with pre-warmed (70deg.C) hybridisation mix.

Wash twice with pre-warmed hybridisation mix for 30 minutes each at 70deg.C.

Wash with a 1:1 mixture of hybridisation mix and TTBS (pre-warmed) at 70deg.C for 20 minutes.

Rinse three times with TTBS. Wash twice with TTBS for 30 minutes each at room temperature.

Block embryos with PBT containing 10% sheep serum for three hours at room temperature.

**C: Antibody Visualization of Digoxygenin**

Incubate with pre-absorbed anti-digoxygenin antibody diluted to a final concentration of 1:2000 at 4deg.C overnight.

Rinse three times with TBST.

Wash three times with TBST for one hour each. If the embryos are large, a further wash overnight at 4deg.C will help cut down background.

Wash twice with Alkaline Phosphatase buffer for one hour each.

For every ml of Alkaline Phosphatase buffer, add 4.5ul of NBT and 3.5ul of BCIP, and develop in the dark for between 2-20 hours, depending on the abundance of the RNA. The product should usually be visible in an hour or two.

When the reaction has proceeded to your satisfaction, it is imperative to quickly stop the reaction to prevent excess background. Wash twice in alkaline phosphatase buffer for five minutes each, and then wash at least three times in PTw **buffered to pH 5.5** for 1 hour each in the dark, and then fix in MEMFA for an hour. After this, the embryos can be cleared in glycerol.

### III: In Situ Hybridization on Cultured Cells

This is basically a modification of the whole-mount protocol, taking into account the requirement for less rigorous washing. It evolved for use on cells cultured in 35mm dishes, but can be adapted to coverslips. In general, the signal tends to be come up much more slowly than in either sections or whole mounts.

#### A: Pre-Treatment of Cells

Fix in 4% paraformaldehyde in DEPC-PBS at room temperature for 10 minutes.

Wash three times in DEPC-PBS at room temperature for 5 minutes.

To 25ml 0.1M triethanolamine, pH8.0, add 62.5ul acetic anhydride and quickly mix until thoroughly dispersed. Incubate cultures in this mixture for 10 minutes at room temperature.

Wash cultures in 1xSSC for five minutes at room temperature.

Permeabilise cultures with 0.2M HCl in DEPC-water for 10 minutes, and wash twice in DEPC-PBS for 5 minutes each.

Pre-hybridize for 6 hours at room temperature. Remove pre-hyb and add probe at a final concentration of between 1 and 2ug/ml. Hybridise overnight at 60deg.C.

**N.B.** To prevent evaporation, incubate in a tight-sealing tupperware box containing towels soaked in 50% formamide and 5xSSC.

#### B: Washing Steps

Rinse cultures in 0.2 x SSC and then wash in 0.2 x SSC at 60deg.C for 1 hour.

Adjust cultures to room temperature in 0.2 x SSC for 5 minutes.

Block cultures in 20% sheep serum in PBT for at least one hour at room temperature.

**C: Antibody Visualization of Digoxygenin**

Incubate cultures with anti-digoxygenin antibody (coupled to alkaline phosphatase) diluted to a final concentration of 1:1000 in 20% sheep serum in PBT at 4deg.C overnight, or for two hours at room temperature. It is not necessary to use pre-absorbed antibody, although it doesn't hurt.

Rinse three times in PBT.

Wash four times with PBT at room temperature for 10 minutes each.

Wash twice in Alkaline Phosphatase buffer (first wash without levamisole, second wash with) at room temperature for 10 minutes each.

For every ml of Alkaline Phosphatase buffer, add 4.5ul of NBT and 3.5ul of BCIP, and develop in the dark for between 2-36 hours, depending on the abundance of the RNA. It may be necessary to wash the cultures and add fresh reaction mixture after 12 hours or so.

When the reaction has proceeded far enough, wash in PBT, and fix in MEMFA.

**Appendix: Additional Techniques**

**A: Preparation of RNAse - Free Slides**

Soak VWR slides overnight in Dichrol at room temperature in a fume hood. Wash the slides thoroughly to remove any residual Dichrol, rinse for one hour in running water, then for a further hour in running distilled water.

Dry slides at 150deg.C for 20 minutes.

Dip slides in a 2% solution of TESTA (3-aminopropyltriethoxysilane; Sigma A-3648)in dry acetone for 5 minutes.

Wash in 2 changes of acetone and three changes of DEPC-water. Dry overnight at 42deg.C and store dry. TESTA slides should be used within 6-8 weeks, as their adhesive properties tend to fade after this time.

**B: Probe Preparation**

Cut between 20 and 40ug of maxi-prep quality plasmid DNA with a five-fold excess of an appropriate restriction enzyme for 2 hours. Check digestion on mini-gel.

Extract cut template in an equal volume of 50 : 48 : 2 phenol : chloroform : isoamyl alcohol. Spin down, transfer the upper layer to a fresh tube and extract with chloroform : isoamyl alcohol.

Precipitate upper layer with 1/9 volume of 3M NaOAc and 2 volume ethanol at -80deg.C. Spin down at 4deg.C for 15 minutes. Wash pellet with 70% EtOH and spin again. Resuspend pellet in 20ul of RNAse-free TE, and store at 4deg.C until required.

Set up the following reaction in 50ul total volume:

5x Stratagene synthesis buffer 10ul
1M DTT (RNAase free) 0.5ul
10mM NTPs/digoxygenin-UTP 2.5ul
RNAsin 10 units
RNA polymerase (T3, T7 or Sp6) 90 units
DNA template 2.5ug
DEPC-water to 50ul

Incubate at 37deg.C for 2 hours.

Remove 2ul for mini-gel sample. Add 20 units of RNAse-free DNAse and continue incubation for a further 10 minutes at 37deg.C. Remove a second 2ul sample and check that DNA has been degraded on a 1% TBE mini-gel.

Add 52ul of 'Stop' buffer to the reaction.

Separate unincorporated ribonucleotides on a Sephadex G50 spin column by spinning for 2 minutes on setting 5 on the Anderson Lab benchtop centrifuge. (Modify as necessary).

Transfer the purified probe to a clean tube. Add 1/9 volume of 3M NaOAc, pH4.8 and 2 volumes of ethanol. Precipitate at -80deg.C for 10 minutes. Spin down at 4deg.C for 15 minutes, wash the pellet in 95% EtOH/5% DEPC-water and spin again for 5 minutes.

Resuspend the pellet in 50ul of an RNAse-free solution of 40mM $NaHCO_3$ / 60mM $Na_2CO_3$. Remove a 1ul sample for OD260 measurement. Incubate at 60deg.C for 35 minutes to hydrolyse the probe into small fragments (between 200-300 bp). 35 minutes works fine for a 1kb probe. For other probe sizes, use the following formula:

The amount of time, *t* , for hydrolysis in 40mM $NaHCO_3$ / 60mM $Na_2CO_3$ at 60deg.C is given by:

(Starting length, kb) - (Desired length, kb)

-------------------------------------------------- = *t*

(0.11) (Starting length, kb) (Desired length, kb)

Precipitate hydrolysed probe again as in (8) above. Resuspend the probe in hybridisation buffer to a final concentration of 10ug/ml. **N.B. Resuspend in a small volume first, then make a final dilution.**

Store probe at -20deg.C until required. Probes should stay stable for months on end.

**C: Sephadex G-50 Spin Column**
Suspend Sephadex G-50 in distilled water. DEPC-treat overnight and autoclave. Let Sephadex settle out, and replace water with RNAse-free 0.3M NaOAc pH 6.0/ 0.1% SDS.

Pack the base of a 3ml syringe with glass wool that has been siliconised with "Sigmacote" and autoclaved. Handle wool with autoclaved forceps.

Load 5ml of G-50 slurry onto the column and spin down. (3 minutes at setting 5 on IEC Clinical benchtop centrifuge - modify as necessary).

Remove buffer from tube and replace with a clean Eppendorf tube. Column is now ready for use.

**D: Pre-absorbing Anti-Digoxygenin Antibody**
Fix a series of rat/chick E12.5 - E13.5 embryos in 4% paraformaldehyde for 2 hours at room temperature. Use about 8 rat embryos for every ml of 1:200 antibody. Wash with PBS and store at -20deg.C in methanol.

Rehydrate in:

75% MeOH : 25% H2O 5 minutes at room temperature
50% MeOH : 50% H2O
25% MeOH : 75% H2O
100% PTw

Dissociate embryos with a syringe and incubate in 10% heat inactivated serum in PTw for one hour at room temperature. Spin down and discard supernatant.

To the minced embryos add an equal volume of 1:200 anti-digoxygenin antibody in PTw containing 1% serum. Incubate for three hours at room temperature on a rotary wheel.

Spin down the embryos, recover supernatant and dilute to 1:1000 with PBT containing 20% serum.

**N.B.** Use a serum species compatible with the antibodies you are using. The Boehringer anti-DIG antibodies are made in sheep.

**E: Pre-absorbing Anti-Digoxygenin Antibody - Embryo Powder Method**
This is an alternative to the protocol above, which may be more convenient for small batches of antibody.

Homogenise embryos of an appropriate age and species in a minimum volume of ice cold PBS.

Add 4 volumes of ice cold acetone, mix well and incubate on ice for 30 minutes.

Spin pellet out at 10,000g for 10 minutes, wash with ice cold acetone and spin down again.

Spread pellet out on filter paper, let it dry thoroughly and grind into a fine powder. Store at 4deg.C.

To produce 2ml of pre-absorbed antibody:

Weigh out 3mg embryo powder and mix with 0.5ml PBT.

Incubate at 70deg.C for 30 minutes. Vortex hard for 10 minutes.

Cool mixture on ice, add 5ul sheep serum and 1ul anti-digoxygenin antibody. Mix for 1 hour at 4deg.C.

Spin down hard and dilute supernatant to 2ml with PBT containing 1% sheep serum.

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## Solutions

**Stop Buffer**
1% SDS
20mM EDTA
20mM Tris pH7.5
100mM NaCl

**PK Buffer:**
50mM Tris-HCl pH 7.5
5mM EDTA
1M Triethanolamine, pH 8.0:
Add 66.5 Triethanolamine and 20ml conc. HCl to 413.5ml DEPC-water in an RNAse-free bottle.

**PTw:**
1xPBS
0.1% TWEEN-20

**PBT:**
1xPBS
2mg/ml BSA
0.1% Triton X-100
100x Denhardt's Solution
2% BSA (ICN 810661)
2% Polyvinylpyrrolidone (PVP-40)
2% Ficoll 400
Make a slurry in DEPC-water and dilute.

**Hybridization Solution:**
For 100ml
50% Formamide 50ml
5x SSC 25ml 20xSSC
1mg/ml Yeast tRNA 2ml of 50mg/ml in DEPC-H2O
100ug/ml Heparin 10mg
1xDenhardt's Solution 1ml 100x
0.1% Tween 20 (Sigma P-1379) 0.1ml
0.1% CHAPS (Sigma C-3023) 0.1g
5mM EDTA 2.5ml 0.2M EDTA pH 8.0
**All components should be RNAse free**

**Cheaper Hybridization Solution for Whole Mounts:**
For 100ml
50% Formamide - 50ml
1.3xSSC (**pH 4.5** with citric acid) - 7.5ml 20xSSC pH4.5
50ug/ml Yeast tRNA - 250ul of 20mg/ml in DEPC-H2O
100ug/ml Heparin - 10mg
0.2% Tween 20 - 0.2ml
0.5% CHAPS (Sigma C-3023) - 0.5g
5mM EDTA - 2.5ml 0.2M EDTA pH 8.0
**All components should be RNAse free**

**NBT:**
75 mg/ml Nitro blue tetrazolium in 70% dimethyl formamide and 30% water

**BCIP:**
50 mg/ml 5-bromo-4-chloro-3-indoyl phosphate in 100% dimethyl formamide

**TBS:**
500mM NaCl
20mM Tris, pH 7.5

TTBS:
500mM NaCl
20mM Tris, pH 7.5
1% Tween 20

MEMFA:
0.1M MOPS pH 7.5

2mM EGTA
1mM $MgSO_4$

**3.7% Formaldehyde**
Make a 10x stock of the salts and add fresh formaldehyde each time.

**Alkaline Phosphatase Buffer:**
100mM Tris, pH 9.5
50mM $MgCl_2$
100mM NaCl
0.1% TWEEN 20
5mM Levamisole - add fresh each time.
N.B. This buffer should always be made up fresh each time from its components - it tends to acidify quite quickly.

**Reagents, Glassware and Apparatus**
- The following solutions can be made up in untreated bottles. Treat with 0.1% DEPC overnight at 37deg.C and then autoclave:
EDTA, NaCl, $MgCl_2$, $NaHCO_3$ / $Na_2CO_3$, NaOAC

- The following solutions cannot be autoclaved. Make these up in DEPC-treated water in glass bottles that have been baked at 170deg.C for 8 hours, and whose caps have been soaked in water containing 0.1% DEPC overnight at 37deg.c and then autoclaved:
Tris, SDS, Denhardt's, any buffers with Tween, Triton or CHAPS, Hybridisation buffer

- The following solutions do not need to be RNAse free:
TBS, TTBS, PBT, BCIP, NBT, MEMFA, Tris-Imidazole buffer
- PTw for whole mounts and culture

## TISSUE PREPARATION FOR IN SITU HYBRIDIZATION

Josiah N. Wilcox

Harvest tissue and rinse in PBS or saline.

Immerse tissue in 4% paraformaldehyde/0.1M sodium phosphate buffer pH7.4 (recipe follows) at 4°C for 1-3hrs. Try to avoid overnight fixation if possible as this causes problems with tissue adherence on slides during the hybridization procedure.

Immerse tissue in sterile 15% sucrose/1xPBS (recipe follows) 3 hrs. to overnight at 4°C.

Embed tissue in O.C.T. (Baxter No. M7148-4), M1 (Lipshaw) or any other convenient embedding matrix for frozen sectioning in plastic embedding molds (. Tissue should be oriented in the block appropriately for sectioning (cross-section, longitudinal etc.). Note the tissue number on the block directly for reference.

Freeze tissue block in liquid nitrogen. Place the bottom third (aproximately) of the block into the liquid nitrogen, allow to freeze until all but the center of the O.C.T. is frozen, and allow freezing to conclude on dry ice.

Store tissue blocks at -70°C in a sealed container or wrapped in foil and ship on dry ice.

It is also possible to use fresh frozen tissue for in situ hybridization if the paraformaldehyde/sucrose method is not feasible. Tissues should be rinsed in saline or PBS and frozen in O.C.T. blocks in liquid nitrogen as outlined above. Although not optimal, it is also possible to use snap frozen material tissue without an embedding matrix. The fixation, sucrose, and O.C.T. steps are used primarily to improve the tissue morphology.

It is expected that the fixation times outlined above will not result in complete fixation of large pieces of tissue. However, the additional fixation step at the beginning of the in situ hybridization procedure should ensure adequate fixation of such tissues prior to hybridization.

This protocol has been used successfully on large (up to 1 cubic cm) and small (1 cubic mm) tissue samples.

**4% Paraformaldehyde**

Mix in a two liter flask:
200ml 0.5M NaPO4, pH 7.4
800ml depcH20
Heat to 70°C with stirring on hot plate in fume hood
Add 40g Paraformaldehyde (EM grade, Polysciences, Cat No. 0380)
Once the solution has cleared (it should take 5 minutes or less), filter with a side-arm flask, Buchner funnel and Whatman No. 2 filter paper.
Immediately pour the solution into a one liter bottle which has been packed in ice. This cools the solution quickly and prevents breakdown of the paraformaldehyde.
Store at 4°C for up to two weeks.

**15% Sucrose in PBS:**
500ml sterile PBS
75g "RNase free" sucrose
Mix above and filter sterilize with a disposable Nalgene filtration unit type S(0.45 micron). Store at 4°C.

**USE OF FISHERBRAND SUPERFROST/PLUS MICROSCOPE SLIDES FOR IN SITU HYBRIDIZATION**

We use Fisherbrand SuperFrost/Plus positively-charged microscope slides (Cat. No. 12-550-15) for all of our frozen tissue sectioning and have very good tissue retention on slides after an in situ hybridization experiment. SuperFrost/Plus slides require no preparation time prior to cryosectioning and are competitive in terms of labor cost and reagent expenses.

**SECTIONING OF FROZEN TISSUES FOR IN SITU HYBRIDIZATION**

Frozen tissues prepared as described can be wrapped and stored for many years prior to sectioning, without loss of the mRNA signal. The biggest problem with stored tissue blocks is that they tend to dessicate if not properly wrapped and the O.C.T. (Optimal Cutting Temperature compound) can be difficult to cut.
Blocks should be removed from the -70°C freezer and allowed to equilabrate with the cryostat chamber temperature. Tissues can be cut at any convenient temperature (-15 to -35°C) as needed. Most tissues cut well at -15°C (brain, kidney, liver, vessels, muscle, etc.) however fatty or more difficult tissues (adipose tissue, skin, lung) require temperatures as low as -35°C or less to obtain good sections. Care should be taken not to touch the face of the slides but handle by the edges only. Frozen sections 5-7µm (thinner is OK but thicker, over 10µm, may present problems for visualizing mRNA in situ) should be cut, thaw-mounted onto room-temperature slides, and immediately refrozen by placing slides with sections into a slide box (VWR micro slide box #48444-003) with a single dessicant capsule (Humi-Cap see below). When the box is full, place the top on the box and store at -70°C. Sections cut and stored with dessicant are stable for in situ hybridization and immunohistochemistry for most antigens for over 5 years.