Revised Stern lab protocol for whole-mount in situ hybridization

Based mainly on the protocol by Domingos Henrique and David Ish-Horowicz, who in turn modified protocols from Ron Conlon, Richard Harland, Phil Ingham and David Wilkinson.

Transcription of DIG-riboprobe

Cut vector with appropriate enzyme as appropriate, 4-5 hours or overnight.

After cutting, run agarose gel with 1/20th. of cutting reaction.

Phenol:Chloroform extract (add an equal volume of Phenol [equil. pH 7.0-8.0*] : Chloroform 1:1, vortex hard, spin on microfuge for 1-2 min).

* DNA goes into the organic (phenol/chloroform) phase at acid pH but into the aqueous phase at alkaline pH.

Take top layer into clean Eppendorf and add 10µl 3M Na Acetate for every 100µl and 2.5x its volume of absolute alcohol. Vortex briefly and put at -20 C. Minimum precipitation at this temperature or lower is about 1 hour. Otherwise leave overnight.

Spin 4 ^{o}C 15 min, take off alcohol. probably won't see pellet but be careful not to touch it.

Wash with 150µl 70% EtOH, vortex, and spin again 5 min.

Take off alcohol carefully and dry pellet at 37 or 65 ^{o}C completely.

Add clean (preferably commercial Ultrapure, without traces of DEPC that may inhibit enzyme reactions) water to a final concentration of about 1mg/ml. Allow to dissolve at 37 or 65 ^{o}C for at least 15 min, with occasional vortexing or flicking the tube.

Transcribe with appropriate enzyme (T3, T7 or SP6). Proportions as follows (add in this order):

Transcription mix:

Component	for 1µg DNA	for 3µg DNA
DNA (1µg/µl)	1 µl	3 µl
Water	15 µl	22 µl
5x transcription buffer	6 µl	10 µl
DIG-nucleotide mix	2 µl	5 µl
DTT (10x)	3 µl	5 µl
RNAsin	1 µl	1 µl
enzyme (T3, T7 or SP6)	2 µl	4 µl

Total:	30µl	50µl

Transcribe at 37 ^{o}C for 2 hours.

NOTE FOR TRANSCRIPTIONS WITH SP6: Use 2-3 times the amount of DNA template shown above for the transcription reaction, and transcribe at 40^{o}C.

Add DNase (1 µl per µg DNA): first make up a stock of 1:10 DNase in transcription buffer with 10% DTT (e.g.: 6 µl water, 1µl DTT, 2 µl transcription buffer, 1µl DNase I), then add this to tube. Incubate 30 minutes at 37 ^{o}C.

At this point, take an aliquot (e.g. 1/20th of sample) and run an agarose gel to check probe.

Make up volume to 80µl with water, and add 8µl EDTA to stop the DNAse.

Add 10µl 4M LiCl and 250µl absolute EtOH. Vortex briefly and put at -20 ^{o}C as above.

Precipitate overnight (min. 1 hour). Spin at 4 ^{o}C 15 min. Take off alcohol (this time you should see a good pellet).

Wash with 300 µl 70% EtOH. Vortex very well. The pellet should be dislodged and should break up into many little pieces, but be careful because it may stick to the upper part of the tube.

Spin again 5 min. Take off alcohol.

Wash again with 50µl absolute EtOH. Spin briefly and remove alcohol.

Allow to dry completely at 37-65 ^{o}C.

Dissolve pellet in water at 1mg/ml approx. (transcription should yield about 8x the original weight of the DNA). Leave at 37-65 ^{o}C for at least 15 min, vortexing occasionally.

Denature probe for 3 min at 95 ^{o}C and immediately cool on ice 5 min.

Spin briefly, then add 10x the volume of hybridization buffer for storage (or make up to about 100-200 ng/ml in hybridization buffer). Store at -20 ^{o}C.

For *in situ* hybridization with 2 probes: transcribe second probe exactly as above, except that instead of DIG-nucleotide mix one uses FITC-labelled UTP (Boehringer) mixed with unlabelled nucleotides to give the same proportions of nucleotides (see Boehringer sheet). **Most important:** for *in situs* with 2 probes, the complete removal of all unbound DIG and FITC is crucial for low background and good discrimination between the 2 probes. There are 2 main ways to achieve this: (a) pass the transcript, after dissolving it in water, through a RNase-free mini-G25 spin column; (b) precipitate each riboprobe twice: after dissolving the pellet in water, phenol-chloroform extract and re-precipitate in LiCl/70%EtOH overnight. In both cases, wash each pellet several times in 70% EtOH (with FITC-probes, at least until all yellowish colour disappears from supernatant).

Note: for in situ hybridization with 2 probes, it is important to use two strongly expressed genes. Label the strongest of these two with DIG and develop it second using INT-BCIP (brick red) or MagentaPhos-TetRed (magenta). Label the less strong one with FITC as above, and develop it first

using NBT-BCIP.

Dissections

Collect embryos in calcium-magnesium-free PBS (CMF).

Fix in freshly made 4% formaldehyde/CMF/EGTA (4% w/v paraformaldehyde powder added to CMF preheated at 65 ^{o}C, stirring continuously. Adjust pH to about 7.5 with 1N NaOH. Allow to cool, then add EGTA to final concentration of 2mM).

Leave in this 1 hour (at room temperature) or overnight at 4 ^{o}C.

Transfer embryos to absolute methanol, and store in this for up to 1 week at -20 ^{o}C. If embryos are to be kept longer before in situ, it is best to take them through to the end of Day 1 (until just before adding the probe) and then keep them in the freezer until needed (this seems to lead to no loss of signal, even if the embryos are kept virtually indefinitely in Pre-hyb). When required, put the embryos into the water bath at the hybridization temperature for a few hours, then add probe as if at the end of Day 1.

First Day: Pretreatments

Rehydrate embryos through 75%, 50% and 25% methanol in PTW (PTW=CMF with 0.1% Tween-20), allowing embryos to settle between changes.

Wash 2x with PTW, 10 min each.

For embryos older than about 2 days, bleach for 1 hour in 6% H_2O_2 (1ml H_2O_2 + 4ml PTW from 30% stock).

Wash 3x with PTW, 10 min each. For the last wash, measure the volume of PTW (use 2 or 5 ml, depending on size of tube).

Add Proteinase K (1:1000; final conc. = 10µg/ml). Incubate at room temp. for 30 min. regardless of stage of embryos, but reduce this to 15 min for New cultured embryos. During incubation, gently roll the tube every few minutes to make sure the sides and top of vial get wet with Proteinase K.

Take off Proteinase K and rinse briefly with a very small volume of PTW (carefully!)

Replace PTW with 4% formaldehyde in PTW (made as above but doesn't need to be fresh), containing 0.1% glutaraldehyde. Postfix 20-30 minutes.

IMPORTANT NOTE ON WASHES: Take off and replace all solutions **with a Pasteur pipette, not a Gilson**. Tilt the vial and turn around to remove solutions with the pipette, without sucking the embryos into it. At each step, remove **all** the solution from vial until embryos stay in position attached to wall, but then add new fluid quickly so they don't dry. When adding new liquid, turn the vial so as to add it to the opposite side of the vial to where the embryos are, and let it run down the wall of the vial. We use 5 ml glass scintillation vials with screw-cap for the whole protocol.

Prehybridization and hybridization

Remove postfixing solution and wash 2x briefly with PTW.

Remove PTW, and replace with 1 ml hybridization solution:

Hybridization solution:

Component (stock conc.)	**Final conc.**	**volume to add**
Formamide	50%	25 ml
SSC (20x, pH 5.3 adjusted with citric acid)	1.3x SSC	3.25 ml
EDTA (0.5M, pH 8.0)	5mM	0.5 ml
Yeast RNA (20mg/ml)	50µg/ml	125 µl
Tween-20	0.002	100 µl
CHAPS (10%)	0.005	2.5 ml
Heparin (50 mg/ml)	100µg/ml	100 µl
H_2O		~18.4 ml
Total:		50 ml

Remove hybridization mix, and replace with another 1-2 ml (5 ml if using large vials).

Place tube upright in a beaker in water bath at 70 ^{o}C. Incubate 2-6 hours.

Remove hybridization mix, and replace with probe in hybridization mix (see above "Transcription").

For probes <~400 nucleotides, it **may** be necessary to use a lower hybridization temperature (e.g. 62-65 ^{o}C for 100-200 nt, 65-68 ^{o}C for 200-350 nt.). Prehybridize at 70 ^{o}C, then lower the temperature when adding the short probe.

For *in situ* hybridization with 2 probes, add both probes simultaneously.

Second Day: Post-hybridization washes

Remove probe **(keep for recycling at least 15 times. it gets better!)**.

Rinse 3x with a small volume (<1ml) prewarmed hybridization solution.

Wash 2x with 1.5 ml (4 ml if large vial) prewarmed hybridization solution, 30 min in water bath.

Wash 20 min with prewarmed 1:1 hybridization solution : TBST (1:10 from the following stock)

10x TBST:

NaCl	8 g
KCl	0.2 g
1M Tris-HCl pH 7.5	25 ml
Tween-20	11 g
H_2O	~64 ml
Total:	100 ml

Rinse 3x with TBST

Wash 3x 30 min with TBST

Block embryos with 5% heat inactivated (at 55 ^oC for 30 min) sheep serum in TBST with 1 mg/ml BSA, 3 hours.

During this time, preabsorb antibody as follows:

(a) weigh X mg of embryo powder, where X=2x number of mls of final vol. of Ab. solution needed into an Eppendorf.

(b) add 500 µl TBST and vortex for 20 seconds

(c) heat to 70 ^oC for 30 minutes, vortex again 20 sec. and spin down at LOW speed for 1 min - just enough to get the powder to form a loose pellet.

(d) suck up the supernatant and discard.

(e) wash the pellet 5 times with 500 µl TBST, spinning low speed each time (this is to remove any fat that may be floating on the supernatant, which both makes a mess later and increases background for some reason). Repeat until no more fat at top of sup't.

(f) resuspend the pellet in 100 µl BLOCKING BUFFER (not TBST) for every bottle of embryos, mixing gently (not shaking). Incubate 10 min. RT. on rocker.

(g) add antibody so that the final concentration will be 1:5,000. For example: if your final incubation in antibody overnight will have 1 ml per tube, your embryos are now sitting in 1ml blocking buffer. If you have 5 bottles, add 1 µl antibody to the 500 µl blocking buffer with the embryo powder.

(h) After absorbing antibody with powder, spin down HARD (high speed) in the microfuge for 3 min. Keep

the supernatant.

Dilute the supernatant with antibody with blocking buffer so that final concentration is 1:5,000.

Remove blocking buffer from embryos and replace with antibody incubation medium.

Incubate overnight at 4 ^{o}C on a rocking platform.

Third Day: Post-antibody washes

Remove antibody solution **(keep at 4 ^{o}C for recycling at least 15 times, it gets better!).**

Rinse 3x with TBST

Wash 3x 1 hour with TBST, rocking (fill vial right up to the top). (Older embryos need more washing)

Wash 2x 10 min with NTMT

NTMT:

5M NaCl	1 ml
2M Tris HCl (pH 9.5)	2.5 ml
2M $MgCl_2$	1.25 ml
10% Tween-20	5 ml
H_2O	44.75 ml
Total:	**50 ml**

Incubate in NTMT containing 4.5 µl NBT (75mg/ml in 70% DMF) and 3.5 µl BCIP (50mg/ml in 100% DMF) per 1.5 ml, rocking, protected from light, at room temperature.

No need to look for the first 15 min. Thereafter look occasionally. Colour may take anything from 15 min to 3 days (or longer!) to develop at room temperature. If you want to go home and colour hasn't quite developed on the first day, leave the vials in the cold room overnight and take them out to room temp. again the next day if necessary. At the end of the 2nd day there is no point in putting them in the cold room again.

After colour has developed as desired, stop by washing 2x 10 min in TBST or PBS.

For *in situ* hybridization with 2 probes, proceed from the last step as follows:

Fix at least overnight or 24 hours (or longer) in 4% paraformaldehyde (**no** glutaraldehyde) at 4 ^{o}C (to inactivate alkaline phosphatase).

Rinse 3x and wash 5x 30min (or more) in TBST containing **0.1% Tween** (rather than 1%) (it is very important to remove all traces of formaldehyde as well as all traces of NBT-BCIP before the next step).

Further inactivate first alkaline phosphatase by incubating 45 min - 1 h in TBST/0.1% Tween at 65-70 ^{o}C.

Wash 3x 15min in TBST/0.1% Tween, and then 3x 15min in TBST/1% Tween (to remove any further traces of NBT-BCIP which are leached out in the cooking above).

Block (as described above for day 2) 2 h in TBST/BSA/goat serum.

Add anti-FITC antibody coupled to alkaline phosphatase, preabsorbed with chick embryo powder, at a final concentration of 1:5,000 as described for the first antibody detection. Incubate overnight at 4 ^{o}C.

Fourth Day: Antibody washes and detection of 2nd probe

Remove antibody solution **(keep at 4 ^{o}C for recycling at least 10 times).**

Rinse 3x with TBST

Wash 3x 1 hour with TBST, rocking (fill vial right up to the top) (more washes for older embryos).

Wash 2x 10 min with NTMT

Then incubate in **EITHER:**

NTMT containing 7.5 µl INT-BCIP (Boehringer) per ml

OR:

NTMT containing 5 µl Magenta Phos (Molecular Probes; 50mg/ml in 100% DMF) and 4 µl Tetrazolium Red (Sigma, 75mg/ml in 70% DMF) per ml,

rocking, protected from light, at room temperature. Continue as described above for first probe (day 3). **NB:** Colour development with INT-BCIP is about the same as with NBT/BCIP (but after 3-4 hours background may start to come up), but with Magenta Phos/Tet Red can take considerably longer, often overnight or several days.

For 1- or 2-probe *in situs*, finish by fixing in 4% formaldehyde and store in this if desired.

To process for histological sections (wax), take fixed embryos and dehydrate/clear as follows:

(a) absolute methanol, 10 min.

(b) propan-2-ol, 5 min.

(c) tetrahydronaphthalene, 30 min.

(d) tetrahydronaphthalene:wax (1:1), 60 ^{o}C, 30 min to 1 hour.

(e) 3x wax at 60 ^{o}C, 30 min.

(f) pour into moulds, allow to set overnight, then section as usual.

Subsequent dewaxing can be done in Histoclear or xylene.

Important note for 2-colour in situs: the reaction product of INT-BCIP does not survive dehydration in alcohols or clearing. For these it is necessary to cut frozen sections in a cryostat or a vibratome. MagentaPhos is OK.

Embryo powder:

Homogenise embryos in a minimum volume of PBS. Add 4x the total volume of ice-cold acetone, and incubate on ice 30 min. Spin 10,000**g** for 10min, throw away supernatant. Wash pellet with ice-cold acetone and re-spin. Spread out pellet and grind it into fine powder on a sheet of filter paper.Air dry the powder, and store it at 4 ^{o}C. It seems to be important that the embryo powder should be made from embryos at approximately the same stages as those being processed for in situ hybridization. Obviously you need more embryos if they are younger.