WHOLE MOUNT IN SITU HYBRIDIZATION
TO DROSOPHILA EMBRYOS WITH RIBOPROBES

Fixation:

Dechorionate embryos in 50% Chlorox.

Wash thoroughly with dH₂O and blot to remove excess liquid.

Fix embryos in a glass scintillation vial, 20 min. with gentle shaking in:

2 ml 4% paraformaldehyde in PBS (freshly prepared)
8 ml heptane

Devitellinize:

Remove lower paraformaldehyde phase completely with a drawn-out
Pasteur pipette.

Add 10 ml of MeOH, shake vigorously 30-60 sec.

Transfer embryos which have fallen to the bottom of the vial
(devitellinized) to a 1.5 ml Eppendorf tube.

(N.B. Devitillenization of early embryos may be improved by removing
the heptane after the paraformaldehyde phase and replacing with 8 ml of
fresh heptane.)

Wash in MeOH, 2-3x over a 10 min. period. Embryos can be stored in MeOH at
-20°C at this point.

Rehydrate into PBST (PBS/0.1% Tween 20), 3-5 min. each step:

7:3 MeOH:PBST
1:1 MeOH:PBST
3:7 MeOH:PBST
100% PBST

Pretreatment:

Wash in PBST, 5 x 5 min.
Treat with non-predigested Proteinase K (Boehringer), 4 µg/ml in PBST, for 3 min., inverting tube several times.

Remove Proteinase K by washing quickly 2 times with PBST.
Wash in PBST, 2 x 5 min.

Refix in 4% paraformaldehyde in PBS, 20 min. with rocking.
Wash in PBST, 5 x 5 min.

**Hybridization:**

Wash embryos in 1:1 HB:PBST, 5 min.

Hybridization buffer (HB):

- 50% formamide
- 5x SSC
- 100 µg/ml sonicated salmon sperm DNA
- 50 µg/ml heparin (SigmaH3125)
- 0.1% Tween 20

Store at -20°C.

N.B. HB used for the prehyb and hybridization should be made as RNase free as possible - in particular, the salmon sperm DNA should be cleaned up by phenol extraction.

Wash embryos briefly two times with 100% HB, then prehybridize at least 2 hr. at 55°C in 200 µl HB.

Heat probe 5 min. at 80°C, then chill on ice. (Probe is generally diluted 1:100 in HB, using 100 µl of diluted probe per sample.)

Remove as much HB as possible from embryos and replace with probe. Mix well by tapping side of tube.

Hybridize O/N at 55°C.

**Antibody Staining:**

Remove probe and wash with 200 µl of fresh HB at 55°C. For best results, it is important to wash for 4-5 hr. with 4-6 changes of HB.

Remove HB, wash 5 min. in 1:1 HB:PBST at RT.
Wash in PBST, 5 x 5 min.
Incubate in 0.5 ml of anti-digoxigenin antibody conjugate, 1:2000 in PBST, for 1 hr. at RT with rocking.

Prior to use, the antibody may be diluted 1:50 to 1:200 in PBST and preabsorbed against fixed embryos (≥100 µl embryos/ml of Ab solution, 4°C O/N). Preabsorbed Ab may be stored for several months at 4°C and diluted to 1:2000 for use. Antibody diluted 1:2000 may be used at least twice.

Wash in PBST, 5 x 5 min.

Wash in developing solution, 2 x 5 min. : 0.1 M NaCl
0.1 M Tris-HCl, pH 9.0
0.05 M MgCl₂
0.1% Tween 20

Transfer embryos to a depression glass dish in 1 ml of developing solution.

Develop with 4.5 µl NBT (75 mg/ml in 70% DMF)
3.5 µl X-phosphate (50 mg/ml in DMF)

Stop reaction by washing well with PBST. Store embryos in PBST at 4°C until ready to embed.

**Embedding:**

Dehydrate embryos in ETOH, 5 min. each step: 30%, 50%, 70%, 90%, 95%, 100%.

Wash in 100% ETOH, 2 x 5 min.

Wash in acetone, 30 sec.

Remove acetone completely with drawn out Pasteur pipette and replace with 200-400 µl Araldite.

Transfer to microscope slide with a cut-off P200 tip, keeping embryos moving to prevent sticking. To prevent crushing of the embryos, break one coverslip into two halves and place each half on either side of the Araldite drop, then place a coverslip on top so that it spans the two spacers.

Polymerize at 65°C O/N.
**Probe preparation:**

The Boehringer Genius 4 kit may be used to synthesize the dig-UTP riboprobe, following the instructions provided with the kit.

After synthesis of the RNA in a 20 µl reaction, add 30 µl dH2O

50 µl 2x carbonate buffer:

120 mM Na2CO3
80 mM NaHCO3
pH to 10.2, store in aliquots at -20°C.

Incubate 40 min., 65°C. This will decrease the size of the probe.

Add 100 µl 0.2 M NaOAC pH 6.0 to stop reaction.

Precipitate RNA by adding 20 µl 4M LiCl
10 µl 20 mg/ml tRNA (phenol/CHCl3 extracted)
600 µl ETOH

-20°C, at least 15 min.

After precipitation, wash pellet with 70% ETOH and resuspend in 150 µl HB. Store at -20°C.

For use, add 1 µl of probe to 100 µl HB, heat to 80°C, 5 min., chill, then add to embryos.