EMBRYO IN SITU HYBRIDIZATION PROTOCOL USING DIG-RNA PROBES

1. Collect, dechorionate, fix, and devittelinate embryos
   Fix either 37% (straight) formaldehyde:heptane for 5 min
   or 7% formaldehyde in PBT:heptane for 25 min. Fixation is important to
   detect mRNA, and you may have to optimize yourself.

2. You can store embryos in ethanol at -20°C indefinitely.

3. When ready to prehybe, transfer embryos to MeOH

4. Rinse with 1:1 MeOH:5% formaldehyde/PBT

5. Rinse with 5% formaldehyde/PBT

6. Post-fix 15 min with 5% formaldehyde/PBT

7. Wash 5 X 5 min with PBT
   important to wash all 5 times and for 5 min; need to get rid of fixative

8. Wash 10 min in 1:1 PBT:hybe buffer

9. Wash 10 min in 1:1 hybe buffer

10. Prehybe at least 1 hour in hybe buffer at 70°C
   Use about 100ul for 30ul volume of embryos
   No need to rock, just put eppendorf in a temp block with water.

11. Add Dig-RNA probe in hybe to embryos
    Again, use about 100ul; just make sure embryos are well covered

12. Hybe overnight at 70°C
    Again, just in the temp block or water bath

13. In morning remove and discard probe and wash:
    1 X 45 min hybe
    1 X 45 min 66:33 hybe:PBT
    1 X 45 min 33:66 hybe:PBT
    4 X 20 min PBT
    All these washes at 70°C, and warm up solutions first; no need to agitate;
    just use the temp block or water bath

    anti-Dig AP from Boehringer Mannheim; no need to preadsorb antibody or
    to block the embryos; rock on Nutator

15. Wash 3 X 20 min with PBT on Nutator
    I've also used 10 min washes
16. Rinse 3 X 2 min with reaction solution:
   100 mM Tris pH 9.5
   100 mM NaCl
   50 mM MgCl₂
   0.1% Tween 20

17. Color reaction:
   Add 4.5ul NBT and 3.5ul X phosphate per ml of reaction solution
   Incubate embryos in this in the dark for 5 min to overnight (often O/N is needed)

18. Stop reaction by washing several times with PBT

Hybe solution:
   50% deionized formamide
   5X SSC
   100 ug/ml sonicated, denatured salmon sperm DNA
   100 ug/ml E. coli tRNA
   50 ug/ml heparin
   0.1% Tween 20
   pH to 4.5 with Citric Acid

NBT = 4-nitro blue tetrazolium chloride
X-phophate = 5-bromo-4-choro-3-indolyl-phosphate
Buy as solutions from Boehringer and store -20°C

Transcribe RNA probe using B.M. Dig labeling kit
No need to fragment the probe, although some people say it helps
Correct concentration of probe found by doing dilution series on embryos
   Usually 1:2000 works for me

For example:
   2ul=1ug cut plasmid DNA
   2ul 10 X reaction buffer (Boehringer)
   2ul 10 X dig-UTP + NTP mix (Boehringer)
   1ul RNAsin (Boehringer)
   1ul T7 or T3 RNA pol (Boehringer)
   up to 20ul with water

   2 hours at 37°C
   run 1 ul of reaction on a gel

   Steps 10-13 (70°C ones) no need to agitate embryos. Just let them sit in a temp
   block or water bath in an eppendorf tube. I usually rock the embryos on a nutator
   through all the other steps. Hybe temp is important: make sure the embryos are
   really at 70°C. If this doesn’t work for your probe, 65°C might be better.