EMBRYO IN SITU HYBRIDIZATION PROTOCOL USING DIG-RNA PROBES

1. Collect, dechorionate, fix, and devittelinize embryos
   Fix either 37% (straight) formaldehyde:heptane for 5 min
   or 7% fomaldehyde 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% formaldeyde/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

14. Incubate embryos in 1:2000 anti-Dig alkaline phosphatase in PBT
    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 $MgCl_2$
    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.