Fix embryos 1 hr in MEMFA (0.1M MOPS; 2mM EGTA; 1mM MgSO4; 3.7% Formaldehyde). Do not over-fix or signal will be reduced. Rinse several times in 1X MEM and dehydrate embryos into MeOH or EtOH. Store at -20°C.

DAY 1

1. Treat baskets (if using) with 50mM NaOH (2g/L) for 20 min.

2. Rehydrate embryos into PTWeen:

5 min	75%, 50% PBS/EtOH
5 min	25% EtOH/PTW
3x 5 min	100% PTW

3. Proteinase K treat:
Dilute 25 mg/ml stock 1:2500 (10ug/ml) in PTW. Treat 2 min (longer times for deep tissue).

4. Rinse 2 x 5 min in PTW

5. Refix in 4% Paraformaldehyde 20 min.

6. Rinse 3 x 5 min in PTW.

7. Prehybridization: change embryos into 1ml hyb solution then 2-4 hrs prehybridization at 60°C.

8. Hybridization in probe overnight (12-16 hrs) at 60°C.

DAY 2

1. Remove probe and do washes at 60°C:
   3 x 20 min 2X SSC
   2 x 30 min 0.2X SSC

2. Wash at RT, 2 x 15 min in MAB.

3. Block 1-2 hrs in MAB + 2% Boehringer Mannheim Blocking Reagent + 20% heat inactivated goat serum (10mls= 2ml 10% BMB + 2ml HIGS + 6ml MAB).

4. Antibody incubation 4hrs RT or 4°C overnight. Use 1:2000 dilution anti-Dig AP (Roche 1093274) or 1:4000 dilution anti-Fluorescein AP (Roche 1426338)

DAY3

1. Wash in MAB: 5 x 60 min at RT. (one can be O/N at 4°C if Ab incubation 4 hrs at RT)

2. Wash in Alkaline Phosphatase buffer: 2 x 5 min.

3. React in AP buffer + 3.5 ul/ml BCIP (50mg/ml) + 4.5 ul/ml NBT (100mg/ml). Allow color to develop 5 min - 1 day (usually 2-4 hours).

4. Stop reaction in postfix (MEMFA) or with MAB washes if continuing on for doubles. One change followed by prolonged fixation (one hour - overnight).

5. Dehydrate embryos in EtOH to get rid of excess substrate.

6. Bleach if embryos are pigmented (9 ml PBS + 1 ml H_2O_2 + 0.5 ml formamide) for several hours under flourescent light. Do only after dehydrating well or embryos will turn purple!