IN SITU HYBRIDIZATION DAY 2: post-hybridization washes, blocking, antibody incubation DAY 3: post-antibody washes DAY 4: color development DAY 1: prehybridization and hybridization NTMT 10.0 mL 5 M NaCl **TBST**0.1% Tween in 1x TBS: 5% methanol/PBT at RT NTMT at RT TBST at RT 50.0 mL 1 M Tris-HCl, pH 9.5 100 mL 10x TBS 25.0 mL 1 M MgCl₂ 00.5 mL Tween-20 001 mL Tween 50% methanol/PBT at RT 899 mL dH₂O dilute to 500 mL with dH₂O hybridization buffer at 68°C 25% methanol/PBT at RT buffer pre-equilibriated at 68°C TBST at RT NTMT at RT PBT at RT PBT at RT solution I 25.0 mL formamide (Sigma) NTMT at RT **6% H2O2 in PBT** 1 mL 30% H₂O₂ 6% H₂O₂ in PBT at RT 12.5 mL 20x SSC, pH 5.0 4 mL PBT 02.5 mL 20% SDS 10.0 mL dH₂O TBST at RT PBT at RT solution I at 68°C buffer pre-equilibriated at 68°C PBT at RT **BCIP and NBT in NTMT** 00.085 mL 50 mg/mL BCIP 00.085 mL 100 mg/mL NBT 24.830 mL NTMT 170 ug/mL BCIP and 340 ug/mL **10ug/mL proteinase K in PBT** 0005 uL 10mg/mL proteinase K NBT in NTMT at RT 10ug/mL proteinase K in PBT at RT cover samples with aluminum foil 4995 uL PBT E08.5 whole embryo 06 mins E09.5 whole embryo (or tail) 10 mins E10.5 whole embryo 12 mins TBST at RT PBT at RT color reaction complete? **4% formaldehyde in PBT** 2.7 mL 37% formaldehyde solution I at 68°C PBT at RT buffer pre-equilibriated at 68°C dilute to 25 mL with PBT wash in PBT at RT (3 x 5 mins) **4% FA, 0.05% GA in PBT** 0541 uL 37% formaldehyde post-fix in 4% FA in PBT 0010 uL 25% glutaraldehyde 4449 uL PBT seal with sticky cover 4% formaldehyde and 0.05% solution III 25.00 mL formamide (Sigma) 05.00 mL 20x SSC, pH 5.0 store at 4°C glutaraldehyde in PBT at RT 00.05 mL Tween 19.95 mL dH₂O TBST at RT solution III at 68°C buffer pre-equilibriated at 68°C PBT at RT PBT at RT hybridization buffer at RT TBST at RT buffer pre-equilibriated at 37°C solution III at 68°C buffer pre-equilibriated at 68°C TBST at RT hybridization buffer at 68°C buffer pre-equilibriated at 68°C solution III at 68°C prepare hybridization solution with probe buffer pre-equilibriated at 68°C 5-10uL DIG-labeled RNA probe per 1mL solution in HB denature probe solution at 80°C TBST at RT solution III at 68°C buffer pre-equilibriated at 68°C TBST at RT **TBST**0.1% Tween in 1x TBS TBST at RT TBST at RT TBST at RT pre-absorb the antibody 10% sheep serum in TBST 0.5 mL 100% sheep serum hybridization buffer with probe at 68°C put 10 mg mouse embryo powder into 2 mL tube with 1.8 mL TBST 4.5 mL TBST heat at 70°C TBST at RT vortex, then cool on ice add 18.0 uL 100% sheep serum add 12.5 uL anti-DIG AP antibody 10% sheep serum in TBST at RT rotation wheel at 4°C cool microcentrifuge to 4°C spin (14,000 rpm) at 4°C TBST at 4°C

antibody solution supernatant diluted to 25 mL with 1% sheep serum in TBST

antibody solution at 4°C

based on Wilkinson and Nieto, 1993 protocol adapted from the lab of Bernhard Herrmann detailed protocol at PGLS Lab Notebook 01, Pages 21 to 25