Whole mount In Situ Hybridization protocol

All reagents prior to hybridization should be RNASE free: Use DEPC treated ddH_2O forsolutions

For Prelimb bud and Limb bud embryos:

Dissect extra-embryonic membranes and open cavities such as the heart and brain to avoid trapping of reagents

Fix embryos in large volume of 4% PFA overnight at 4ºC

Wash embryos 2X 5 min each with PBT (PBS/.1% Tween-20) at RT

DAY1:
Dehydration: Wash for 5 min each at RT with 30%, 50% and 80% MeOH/PBT, then 2X with100% MeOH (Embryos may be stored in 100%MeOH at -20ºC)

Rehydration: Wash embryos for 5 min each at RT with 100% MeOH, 80%, 50% and 30%MeOH/PBT at RT

Wash 2x5 min each with PBT at RT

For Limb bud embryos:

Bleach embryos in 6% H_2O_2 in PBT for 1 hr at RT
Wash with PBT at RT 3X 5min each
Treat with proteinase K at 10ug/ml for 10-20 minutes depending on stage of embryo
Wash 2X 5 min with freshly made 2mg/ml glycine/PBT at RT
Wash 2X 5 min with PBT at RT

For Prelimb embryos:
Permeabilize embryos with 3X 30min washes with RIPA buffer at RT
RIPA buffer:
150mM NaCl
1%Nonidet P-40
.5% deoxycholate
.1% SDS
1mM EDTA
50 mM Tris-HCl
final pH 8.0

For both prelimb and limb bud embryos:
Refix the embryos with fresh .2%GDA/4%PFA in PBT for 20 min at RTWash embryos 4X 5 min each with PBT at RT

Wash once with prehybridization buffer 5 min at RT

Prehybridization Buffer:
50% Formamide
5 X SSC pH4.5
1% SDS
50ug/ml total yeast RNA (boil for 5 min)
10ul/ml ssDNA (boil for 5 min)
50ug/ml heparin
pH 4.5
Incubate embryos with prehybridzation buffer at 70ºC for 1 hr or longer
Hybridize embryos in hybridization solution ON with rocking at 70ºC
Hybridization sol: prehyb with 1ug/ml of DIG-labeled RNA probe (heat probe 5 min)

Day2:
Wash 3X30 min each at 70ºC with solution 1
Solution 1: 50% formamide
5X SSC pH4.5
1% SDS
pH: 4.5
Wash 3 x 30 min each at 65ºC with solution 2
Solution 2: 50% formamide
2X SSC pH 4.5
pH 4.5

Wash 3X 5 min each with TBST plus 2 mM levamisole
10X TBST:
1.4 M NaCl
27mM KCL
.25M Tris-HCL, pH 7.5
1% Tween-20
ddH_2O autoclave

Preblock embryos in TBST plus 10% heat-inactivated sheep serum for 2.5 hrs at RTSheep serum is heat inactivated at 70ºC for 30 min before use

During this step, preabsorb antibody as follows:
Add .5 ml TBST to 3mg of embryo powder and heat for 30 min at 70ºC. Vortex to mix.Cool on ice and add 5ul sheep serum and 1 ul anti-DIG-AP conjugated antibody
Shake gently at 4ºC for 1 hr, spin in microcentrifuge at 4ºC for 10 min
Recover supernatant and dilute to 2 ml with 1% sheep serum in TBST

Discard preblocking solution and add 1ml pre-blocked antibody to embryos for 1-2 min
Replace with fresh preblock antibody and rock at 4ºC ON

Day 3
Wash embryos 3X 5min at RT with TBST
Wash 5X for 1.5 hrs each time at RT with TBST
Wash ON with TBST

Day 4
Wash 3X 10 min each with fresh NTMT
NTMT
100mM NaCl
100mM Tris-HCl, pH 9.5
50mM MgCl
.1%t Tween-20
2mM levamisole

Incubate with detection solution: NTMT plus .25mg/ml nitoblue tetrazolium(NBT) and .13mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium (BCIP)

After detection complete wash embryos 2X with NTMT, once with PBT (pH 5.5)Post fix with 4% PFA/.1%GAD in PBT
Embryos can be cleared through series of 30%,50%,70% and 80% glycerol in PBT