

Whole mount In Situ Hybridization protocol

All reagents prior to hybridization should be RNASE free: Use DEPC treated ddH₂O for solutions

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 4°C

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 with 100% MeOH (Embryos may be stored in 100%MeOH at -20°C)

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% H₂O₂ 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 RT

Wash 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 prehybridization buffer at 70°C for 1 hr or longer

Hybridize embryos in hybridization solution ON with rocking at 70°C

Hybridization sol: prehyb with 1ug/ml of DIG-labeled RNA probe (heat probe 5 min)

Day2:

Wash 3X30 min each at 70°C with solution 1

Solution 1: 50% formamide

5X SSC pH4.5

1% SDS

pH: 4.5

Wash 3 x 30 min each at 65°C 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

ddH₂O autoclave

Preblock embryos in TBST plus 10% heat-inactivated sheep serum for 2.5 hrs at RT

Sheep serum is heat inactivated at 70° C 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 70°C. Vortex to mix.

Cool on ice and add 5ul sheep serum and 1 ul anti-DIG-AP conjugated antibody

Shake gently at 4°C for 1 hr, spin in microcentrifuge at 4°C 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 4°C 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% Tween-20

2mM levamisole

Incubate with detection solution: NTMT plus .25mg/ml nitoblue tetrazolium(NBT) and .13 mg/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