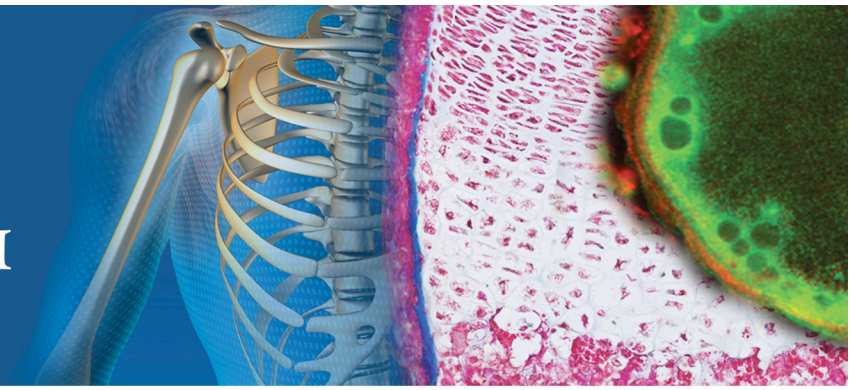

DIG Labeled *In situ* Hybridization Protocol

Tissue Preparation and Sectioning

Use only Milli-Q water to make solutions and RNAse Away treat everything including beakers, graduated cylinders, and forceps. After RNAse Away treatment, rinse only with Milli-Q water.

Paraffin Sections

- Dissect embryos in cold PBS.
- Remove limbs and fix O/N in 4% PFA/1XPBS or 10% buffered Formalin rocking at 4°C (~10 mls per vial).
- Wash in 1XPBS a few times for 3-5' each (~10mls per vial).
- Dehydrate in a series of EtOH rocking at 4°C (10 mls per tube):
  - 70% EtOH 5' (At least)
  - Send to Histology Core for processing or do yourself.
  - 95% EtOH 5' (At least)
  - 95% EtOH 5' (At least)
  - 100% EtOH 5' (At least)
  - 100% EtOH 5' and store at 4°C

- Place limbs in glass container fully covered in xylene 2X 30' to 45' at RT.
- 1X 30' of xylene and liquid paraplast 1:1 ratio in 60°C oven.
- 2X 60' liquid paraplast in vacuum oven (~60°C at –10 in.Hg).
- O/N fresh liquid paraplast in vacuum oven at same settings.
- Embed forelimbs with palm facing up and hindlimbs with the foot sideways. Use metal casting trays and tan biopsy cassette (Fisher Cat# 22038132).
- Store blocks at 4°C until needed.
- Section limbs at 6 μm and put 1 section per slide. Slides should be SuperFrost Plus (VWR 48311-703 or Fisher Cat# 22034979) slides.
- Float sections in water bath at 37-40°C, collect on slides, and store in slide boxes (Fisher Cat# 034482).

Linearization of Plasmid DNA and Probe Preparation

Linearization of Plasmid

Setup the following digestion:

140 µl Milli-Q water
30 µl Plasmid DNA from fresh miniprep
20 µl Appropriate restriction enzyme 10X buffer
2 µl 100X BSA
8 µl Appropriate restriction enzyme (at least 50+ units)
200 µl Total volume

- Incubate sample in 37°C water bath for 4 hours to O/N.
- When digest is finished, purify using Qiagen Qiaquick PCR purification spin columns.
- Run 1 µl of sample on a gel to verify recovery. DNA must be completely linear in order to proceed to Probe Preparation.
- Use about 4 µl per *in vitro* transcription (IVT) reaction.

Probe Preparation

- As per Radioactive In Situ Hybridization Protcol

Preparation of Slides for Hybridization

Be gentle with slides and try not to agitate solutions too much.

- Bake slides at 60°C on slide warmer for 1 hour.
- Allow slides to come to RT (At least 15-20').
- Dewax slides in xylene 2X for 8'.
- Rehydrate slides to DEPC-PBS.
    - 100% EtOH 3'
    - 100% EtOH 3'
    - 75% EtOH 3'
    - 50% EtOH 3'
    - PBS (pH 7.4 - 7.0) 3'
    - PBS (pH 7.4 - 7.0) 3'

Pre-Hybridization and Hybridization

Clean all glass histology dishes, lids, and carriers the day of use with RNAse Away and Milli-Q water and dry. All prehybridization solutions used should be in 600 ml volumes in glass dishes. See Supplies and Solutions for recipes.

- 4% PFA/1X PBS for 15'.
- Rinse 2X in 1X PBS for 3' each.
- Proteinase K (10 μg/ml) in 1X PBS for 15'.
- 4% PFA/1X PBS for 10' (Re-use PFA from first post fix).
- Rinse 2X in 1X PBS for 3' each.
- Acetylation for 10'. (7.8 ml of TEA [Sigma T-1377] added to 12.2 ml of Milli-Q water in a 50 ml Falcon tube, shake and add to 575 ml of Milli-Q water in a glass histology dish, and pH to 7.5. Incubate slides in TEA for 1'. Add 1.8 ml of Acetic Anhydride [Sigma A-6404] to 3.2 ml Milli-Q water in a 50 ml Falcon tube and shake vigorously, then pour in TEA immediately and mix by moving slide rack back and forth. Incubate slides for 10' at RT.)
- Rinse 2X in 1X PBS for 3' each.
- Air-dry slides for 30' (Make sure they are completely dry).
- Add 80 μl of prewarmed (70°C) hybridization solution to each slide and cover with coverslip. (Cut coverslips from hybridization bags).
- Place slides in humidified *in situ* hybridization chamber and prehybridize slides for 1+ hours at 70°C. (Use 50% Formamide for chamber solution).
- Add 1 μl of DIG labeled probe to prewarmed hybridization buffer for each section necessary [ie. 4 sections = 4μl of probe in ~350 μl of hybridization buffer).

- Take off old coverslips, add 80 µl of prewarmed probe per section, and add new coverslips to each section.
- Hybridize O/N in hybridization chambers at 70°C (16-18 hours).

Post-hybridization Washes

Prewarm all wash solutions by microwaving (3 min for 65°C, 1 min for 37°C). Add Formamide after microwaving solution. All washes done in 500 ml glass histology dishes.

- Gently remove coverslips and rinse in 5XSSC at RT.
- Wash in 1XSSC/50% Formamide for 30' at 65°C by placing histology dish in water bath (make sure not to get any water in the dish).
- Wash in TNE for 10' at 37°C in incubator.
- Incubate for 30' in TNE + RNAse A (10 µg/ml) at 37°C. (Add 300 µl of 20 mg/ml RNAse A solution to 600 ml TNE).
- Wash in TNE for 10' at 37°C.
- Wash in 2XSSC for 20' at 65°C in water bath.
- Wash in 0.2XSSC for 20' at 65°C in water bath.
- Wash in 0.2XSSC for 20' at 65°C in water bath.

Antibody Incubation and Detection

Blocking, antibody incubation, and color reaction should be performed in humidified chambers (Pyrex glass dish partially filled with water, slides placed on top of Eppendorf racks to keep them dry, and entire dish covered in plastic wrap. They will then also be covered in aluminum foil for the color reaction.).

- Wash in MABT 2X for 5' at RT.
- Circle all sections with ImmunoPen.
- Block in 20% sheep serum / 2% Boheringer blocking reagent / MABT for 1 hour at RT (1 ml NSS / 1 ml 10% BBR / 3 ml MABT)(Use 90 µl per slide – good for 50 slides).
- Dump off blocking solution and incubate DIG-AP antibody (1:2500 dilution) in 5% sheep serum / MABT O/N at 4°C in humidified chamber. (500 µl NLS / 4 µl DIG-AP antibody / 9.5 ml MABT)(150 µl per slide).
- Wash in MABT 3X for 5' at RT.
- Wash in NTM for 10' at RT.

- Incubate in NTM + NBT + BCIP with 90 µl per slide. (Use 2.5 µl of each NBT and BCIP per 1 ml of NTM)(5 ml is enough to cover 50 slides) (Make just before use).
- Develop slides in dark with humidified chamber covered in aluminum foil. Developing slides can take from a few hours to 3 days.
- Rinse slides in NTM.
- Wash in PBS 2X for 5'at RT.
- Place slides in 4% PFA/PBS for 30+' at RT.
- Wash in PBS 2X for 5'at RT.
- Rinse in Milli-Q water.
- Counterstain with Nuclear Fast Red, dehydrate up EtOH gradient, and coverslip using Vectamount mounting media.

Supplies and Solutions

Sheep Serum or Lamb Serum (heat inactivated)
DIG-AP antibody (Roche 1093274)
Boehringer Blocking Reagent (Roche 1096176) Make 10% stock aliquots.
BCIP (Roche 1383221)
NBT (Roche 1383213)

1X PBS	Dilute from 10X PBS stock.
4% PFA/1X PBS	500 ml 1X PBS microwave for 3' in RNAse Away treated beaker. Add 20g of PFA (Sigma P6148) and adjust to pH 7.4 –7.1 with 10N NaOH using litmus pH paper. Might need to provide additional heat.
5X SSC	125 ml 20X SSC Fill to 500 ml with Milli-Q water.
1X SSC/50% Formamide	25 ml 20X SSC Fill to 250 ml with Milli-Q water and heat in microwave for 3'. Add 250 ml Formamide (Sigma F7503)
2X SSC	50 ml 20X SSC Fill to 500 ml with Milli-Q water.
0.2X SSC	10 ml 20X SSC Bring to 1L with Milli-Q water.

TNE	100 ml of 5M NaCl 10 ml of 1M Tris-HCl pH 7.5 2 ml of 0.5M EDTA Bring to 1L with Milli-Q water.
MABT	11.6 g of Maleic Acid 30 ml of 5M NaCl Add 800 ml Milli-Q water Add 8 g of NaOH pellets and then adjust pH to 7.5 with 10N NaOH. Bring to 1L with Milli-Q water and add 1 ml Tween-20. Need at least 3 L per experiment.
NTM	100 ml of Tris-HCl pH 9.5 20 ml of 5M NaCl 50 ml of 1M $MgCl_2$ Bring to 1L with Milli-Q water. Make sure to pH the Tris each time.