Digoxigenin-Labeled *In Situ* Hybridization for E15.5 Kidneys

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The *in situ* hybridization methods were developed by Dave Wilkinson from protocols from Phil Ingham, Ron Conlon, Barry Rosen, and Richard Harland in David Wilkinson, ed., In Situ *Hybridization: A Practical Approach,* Oxford: IRL Press, 1992.

Reagent Testing – In conducting a large scale, continuous screen, it is critical to fully test each reagent to avoid unnecessary disasters and maintain data consistency. New batches of Proteinase K, antibody, and lots of BM purple need to be carefully tracked. Testing is best done with small numbers (6-12) of wildtype UGSs. Making reagents in large tested batches is ideal. The sources of reagents should also be constant as we have had SDS from a different company cause background issues.

Dissection, Fixation, and Dehydration of UGS Samples

Store samples in 20 ml screw top scintillating vials during dissection, fixation, dehydration, and "Day One" treatment. All wash volumes are approximately 15 ml per scintillating vial.

- Dissect UGS into PBS.
- Remove **all** of liver and intestine and as much membranous tissue as possible.
- Acceptable samples contain two intact kidneys, both gonads, both adrenal glands, and minimally damaged connective tissue, ureter, and bladder.
- Fix in 15 ml 4% paraformaldehyde/PBS at 4°C for 16-18 hours.
- Wash 2x in PBS at room temperature for 10 minutes.
- Wash samples in each of the following at room temperature for 5 minutes:
    - 25% MeOH/0.85% NaCl solution.
    - 50% MeOH/0.85% NaCl solution.
    - 75% MeOH/0.85% NaCl solution.
- Wash 2x in 100% MeOH at room temperature for 10 minutes.
- Store in 100% MeOH at -20°C for up to 6 months.

"Day One" – UGS Sample Preparation

- Rehydrate samples in each of the following at room temperature for 5 minutes:
    - 75% MeOH/0.85% NaCl solution.
    - 50% MeOH/0.85% NaCl solution.
    - 25% MeOH/0.85% NaCl solution.
    - 2x PBT.

- Bleach in 6% hydrogen peroxide in PBT at room temperature for 30 minutes.
    - Wash 3x in PBT at room temperature for 5 minutes each.

- Treat with 10 µg/ml **Proteinase K** in PBT (Boehringer 161519).
    - Timing is important.
    - Do not shake or rotate vial, but gently mix every 5 minutes.
        - E14.5 UGS for 10 minutes.
        - E15.5 UGS for 30 minutes.

- "Quick" gentle wash in 2 mg/ml glycine/PBT (filtered).
    - Wash 2x in PBT at room temperature for 5 minutes each.

- Fix in 0.2% glutaraldehyde/4% PFA in PBT at room temperature for 20 minutes.
    - Wash 2x in PBT at room temperature for 5 minutes each.

- Place in **pre-hyb solution** and heat at 70°C for one hour.
- Store in **pre-hyb solution** at -20°C for up to 1 month or hybridize immediately.

Note: The 30 minute E15.5 **Proteinase K** time provides the best deep probe penetration. We tested 15, 20, 30, and 60 minute treatments with Wnt7b and verified with deep structure markers Hoxb7, Wnt7b, and Shh probes. These times did not affect superficial staining as shown by Wnt11, BF2, and Six2 probes.

Hybridization

Note: Heat probes and samples at 70°C prior to procedure to dissolve hyb solution precipitation. Gently mix and quickly centrifuge probes prior to use.

- In a 48-well plate, add one female and one male UGS per well.

- In a separate 48-well plate, dilute probes to working 1x concentration (0.50 ng/µl) with pre-hyb solution.

    - Total well volume is 200 µl (10 µl 20x probe in 190 µl pre-hyb).
    - Heat plate of diluted probe to 80°C for 5 minutes prior to use.

- Remove pre-hyb solution from wells (at most, from one row at a time).
- Add warm diluted probe to wells.

- Place folded paper towel over 48-well plate. Moisten towel with 15 ml 50% formamide / 50% 10x SSC solution. Wrap in saran wrap. Place in a plastic bag.

- Hybridize for 18 hours at 70°C.

Note: We tested 0.25, 0.50 and 1.0 ng/µl probes and found no difference in appearance or timing of expression patterns (tested with Slc34a1 probe, a late renal tubule marker). Also, we have found that 200 µl of probe is sufficient to hybridize 2 E15.5 UGS and 2 E10.5 embryos in a single well.

Post-Hybridization Washes and Development

Note: All post-hybridization washes are performed with a Hülle & Hüttner BioLane HTI. This is an overview of the typical wash procedure. The BioLane HTI can process 24-192 wells in a single program. The accompanying chart provides the solution formulas and volumes for use with the BioLane HTI. Detailed HTI operation instructions are attached.

- Place BioLane intake tubes in a large flask of clean water; run cleaning program.
- Load samples with plastic transfer pipette into nylon-mesh BioLane plate in a tray containing warm Solution I.
- Load tray into BioLane System 1 or System 2.
- Place intake tubes into solutions. Consult accompanying BioLane guide.
- Place output tube into a hazardous waste jug (formamide waste).
- Do **not** have MBST tube in place for first cycle (tray is already full).
- Start appropriate program; wait for first intake cycle; replace MBST tube.
- Remove hazardous waste jug after final Solution III wash (approximately 4 hours from start time).

- The HTI machine uses 40 ml solution per wash in a half tray; 75 ml solution per wash in a full tray; 150 ml solution per wash in a double tray; and 300 ml solution per wash in a double system, double tray procedure.

Overview of Wash Procedure

- Wash 4x with Solution I at 70°C for 15 minutes each.
- Wash once with 1:1 mixture of Solution I to Solution II at 70°C for 10 minutes.
- Wash 3x with Solution II at room temperature for 5 minutes each.
- Wash with 100 µg/ml RNase A (Sigma R 6513) in Solution II at 37°C for one hour.
- Wash once with Solution II at room temperature for 5 minutes.
- Wash once with Solution III at room temperature for 5 minutes.
- Wash 4x with Solution III at 65°C for 15 minutes each.
- Wash 3x with MBST at room temperature for 5 minutes each.
- Wash in 10% HISS blocking solution at room temperature for 4 hours.
- Incubate in anti-digoxigenin alkaline phosphatase 1:5000 diluent (Roche 1093274) in 1% HISS blocking solution at 4°C for 12 hours.
- Wash 3x with MBST at room temperature for 5 minutes each.
- Wash 10x with MBST at room temperature for one hour each.
- Wash 8x with MBST at 4°C for 2-5 hours each.
- Samples can be held in MBST at 4°C for up to 2 days.
- Wash 3x in NTMT at room temperature for 5 minutes each.

Note: We tested anti-body dilutions of 1:2000, 1:4000, 1:5000, 1:6000, 1:7500, 1:8000, and 1:10000 and found no difference in signal intensity and background among the various dilutions (tested with Slc34a1, Nr1f3, and Tcea3 probes).

Solution Guide (attached)

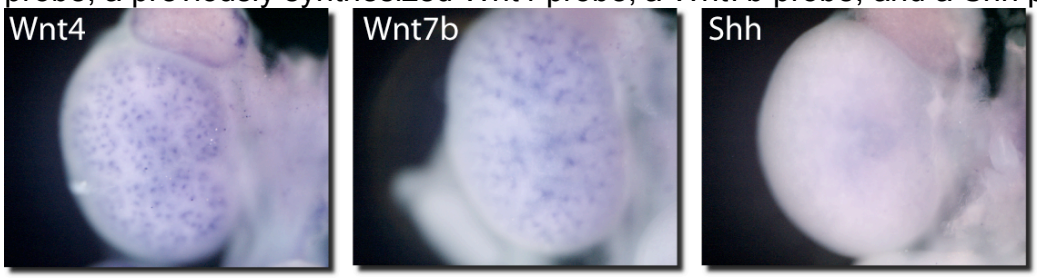
Microsoft Office
Excel Worksheet

Development, Fixation, and Preparation for Data Collection

- Transfer samples from mesh plate to 48-well plate.
- Remove NTMT and add 500 µl BM Purple (Roche 1442074).
    - First, spin BM Purple at 2000 rpm for 5 minutes to remove precipitate.
    - After adding BM purple, wrap well-plate in aluminum foil.
- Monitor samples at 3, 6, 9, 12, 24, 36, and 48 hours.

Note: We use three control samples to verify the consistency of development time between experiments. These controls are Wnt4, Wnt7b, and Shh. These probes generally express at 12, 24, and 48 hours, respectively.

The success of the probe synthesis reaction, the hybridization, and the post-hybridization washes is controlled with four probes: a newly synthesized Wnt4 probe, a previously synthesized Wnt4 probe, a Wnt7b probe, and a Shh probe.



Wnt 4: Strong expression(12 -24 hours).
Wnt7b: Medium expression (24-48 hours)
Shh: Weak expression (48 hours)

- Stop at the emergence of a strong signal or when background begins to appear; maximum 24 hours.
    - Transfer to a new 48-well plate.
    - Wash 3x with PBT pH 4.5 at room temperature for 5 minutes each.
    - Fix in 0.2% gluteraldehyde/4% PFA/PBS at room temp for one hour or 4°C overnight.
    - Hold samples in fix at 4°C until all development has completed.

- Wash 3x with PBS at room temperature for 5 minutes each.
- Wash with 50% glycerol in PBS at room temperature for 4 hours.

- Wash with 80% glycerol in PBS at room temperature for 4 hours.
- Wash with 80% glycerol in PBS at room temperature for 16 hours.
- Photograph data before switching into the glycerol with azide.
- Store in 0.1% azide/80% glycerol/PBS at 4°C in 2 ml screw-top vial (Sarstedt 72.694.006).

Note: Shh, a very weak probe, clearly shows expression pattern after 48 hours. For this reason we chose 24 hours as the maximum BM Purple development time.

Photography

- We photograph samples with a Nikon DXM1200 digital camera attached to a Nikon SMZ1500 scope with 10x eyepiece and 1x optical.
- 3 photos per gene: 3x of male and female UGS each, and 7x close-up of a kidney from the male or female UGS.
- Naming protocol is "YYMMDDi[type][initial]_###". For example, the eighth photograph of a whole mount *in situ* hybridization sample taken by John Harvard on May 15, 2001 is called "010515iWMjh_008".

- Fill a 35 mm Petri dish with 80% glycerol/PBS. Carefully place one sample in center of dish. Avoid introducing debris or air bubbles.

- The light sources (two fiber optic cables) rest nearly parallel and touch the Petri dish on each side, amplifying illumination and minimizing shadows.

- Adjust white balance so that the color of the digital image matches the color of the real image. The center of the bladder generally provides a good white reference point. Non-expressing regions can also provide a white reference.

- 3x zoom exposure time should be from 1/200 to 1/60 of a second. 7x exposure time should be from 1/75 to 1/25 of a second. Shorter exposure times minimize the appearance of debris and air bubbles and produce a sharper image.

- Focus lens on center of the kidneys. The expression pattern is the primary target.

- Record filename, gene name, sex, zoom, and BM Purple development time.