O'Brien Center For Benign Urologic Research
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FLOURESCENT IMMUNOSTAINING OF FORMALIN- FIXED, PARAFFIN EMBEDDED (FFPE) TISSUE SECTIONS

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Reference: Abler LL, Keil KP, Mehta V, Joshi PS, Schmitz CT and **Vezina CM** (2011). A High Resolution Molecular Atlas of the Fetal Mouse Lower Urogenital Tract. ***Dev Dyn*** 240:2364-2377. PMC3177421.

All steps should be conducted with gentile agitation on an orbital shaker, unless otherwise instructed.

Day 1

1. Heat slides to 65°C for 5 min to remove wrinkles and increase tissue adhesion to slide

2. Remove paraffin from slides:
    A. 3 min in Xylene (Fisher #X3P-1GAL)
    B. 3 min in 100 % ETOH
    C. 3 min in 75% ETOH
    D. 3 min in 50% ETOH

3. Epitope recovery:
    A. Immerse slides in 500 ml 10 mM citric acid pH 6.0.
    B. Microwave on Power 50 for 20 min to raise the temp to about 95°C
    C. Cool until lukewarm and proceed to step 5

4. Use wipe to create a dry rectangle around your section

5. Outline samples with hydrophobic pen (Super HT PAP pen, RPI Corp # 195505, being careful to mark only the dry part of the slide)

6. Pipette TBSTw onto the outlined region and let sit for 5 min at 25°C with no agitation so that barrier dries

7. Remove TBSTw and block for 1 hr at 25°C with gentle agitation in TBSTw containing 5% goat serum, 1% BSA, and 1% Roche Blocking Reagent (diluted from 10% blocking reagent stock, prepared in maleic acid buffer, according to manufacturer's instructions. Roche Applied Sciences # 11 096 176 001). This blocking buffer is named RGBT. Alternatively, samples can

be blocked or in another buffer that contains 5% serum from the animal in which the secondary antibody was made

8. Apply primary antibody, diluted in RGBT, and incubate overnight at 4°C

Day 2

1. Wash 5 min in TBSTw at 25°C (repeat 5X)
2. Add Fluor-conjugated secondary antibody (diluted in RGBT) and incubate for 60 min at 25°C in a light protected box
3. Remove secondary antibody solution from slides and wash with TBSTw for 5 min at 25°C in light protected box. Repeat this step 7X. If not staining cell membranes skip to step #20
4. Wash with TBSTw for 5 min at 25°C in light protected box (repeat 2X)
5. Add to slides TBSTw containing DAPI (300 nM final concentration in dimethylformamide, Invitrogen #D3571) and incubate for 5 min at 25°C in light protected box
6. Wash with TBSTw for 5 min at 25°C in light protected box (repeat 2X)
7. Add a small bead of antifade-mounting media (see recipe below) to slide and add cover glass (Corning #2865-18). Image ASAP

End immunofluorescence protocol.

MATERIALS

Tris-Buffered Saline Buffer Containing 1% Tween-20 (TBSTw)
Makes 500 mL of 10X stock solution

- 0.2M Tris-HCl (Fisher Scientific #BP153-1)
- 1.5M NaCl (Fisher Scientific #BP358-212)
- Adjust volume to 500 mL
- Adjust pH to 7.4
- Sterilize by Autoclaving
- Add 1% (v/v) Tween-20® (Fisher Scientific #BP337-100)
- Add sodium azide to a final concentration of 200 µM
- Store at 25°C for up to 6 months

Citric Acid Solution
Makes 50 mL of a 100X solution

- 1M Sodium Citrate Dihydrate (Fisher Scientific #BP327-500)
- Dilute with water, adjust pH to 6.0, store at 4°C for up to 6 months

Blocking Reagent
Makes 200 mL of a 10% solution

- 100 mM Maleic Acid (Sigma Aldrich #M0375-500g)
- 150 mM Sodium Chloride (Fisher Scientific #BP358-212)
- Adjust pH to 7.5
- Qs to 200 mL with ultrapure H2O
- Add 20g Blocking Reagent (Roche Applied Science #11096176001)
- In a 250 mL Erlenmeyer flask in microwave, heat at power 30 for about 3 minutes. Watch carefully and swirl often to prevent boil over
- The buffer will retain a slightly cloudy appearance even when it is completely dissolved
- Aliquot at 10 and 50 mL and store at -20°C for up to 12 months

Notes:

- This buffer is used for reducing non-specific binding of antibody to tissue
- The buffer is very strong and it will take a large amount of NaOH to increase the pH to 7.5. Start by adding 10 solid pellets of NaOH, allow the pH to equilibrate, and then add more pellets or concentrated aqueous 5M NaOH as needed. When you approach pH7.5, the pH will change rapidly in response to added acid/base. Be careful not to overshoot!

Roche Blocking Reagent + Goat Serum + Bovine Serum Albumin + Tween 20 Blocking Buffer (RGBTw) Blocking buffer
Makes 100 mL of 1X solution

- 10 mL Blocking Reagent
- 5 mL Goat Serum (Sigma Aldrich #G6767)
- 1 gm Bovine Serum Albumin Fraction V (Fisher Scientific #BP2600-100)
- 85 mL TBSTw
- Aliquot and store at -20°C for up to 12 months

Notes:

- Used to pre-block and block samples during IHC
- Should be used only if secondary Antibody used in IHC is made in goat serum

Antifade mounting medium recipe (From Jackson ImmunoResearch):

Make a 20% stock solution of n-propyl gallate

- 0.4 grams n-propyl gallate (MP Biomedicals #1-2747)
- 2 ml dimethylsulfoxide (Sigma Aldrich #D8418-50ML)
- Prepare 100 microliter aliquots and store at -20°C for up to 12 months

Make 10 ml solution of antifade mounting medium

- 0.5 ml 20X Phosphate Buffered Saline without Calcium or Magnesium (MP Biomedical #1760420, purchased through Fisher Scientific).
- 9 ml Glycerol (Sigma Aldrich #G5516-500mL)
- Add 100 µl 20% n-propyl gallate dropwise with stirring
- Store solution at 4°C for up to 2 months

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