Immunohistochemistry – Paraffin Section v1

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.

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Materials

REAGENTS

- Blocking reagent (Roche #11096176001)
- Sodium citrate dihydrate (Fisher Scientific #BP327)
- Coverglass, 18x18mm square, #1 (Corning #2845-18)
- 4',6-diamidino-2-phenylindole, dilactate (DAPI; Invitrogen #D3571)
- Dimethylformamide (DMF; Sigma #D4551)
- Dimethyl sulfoxide (DMSO; Fisher #BP231)
- Dulbecco's phosphate buffered saline, modified, without calcium and without magnesium (PBS; MP biomedical # 1760420)
- Ethanol (EtOH; Pharmco-Aaper #111000-200E200G)
- Glycerol (Sigma G5516-500mL)
- Maleic acid (Sigma # M0375-500G)
- Sodium chloride (NaCl; Fisher BP358-212)
- n-propyl gallate (MP Biochemicals #102747)
- Phosphate buffered saline, Dulbecco's, without calcium or magnesium (PBS; Fisher Scientific #SH3001304)
- Pyrex glass dish (Corning 222-R)
- Sudan Black B (VWR #ICI5208810)
- Super HT PAP pen (RPI Corp # 195505)
- Tris hydrochloride (Tris-HCl; Fisher BP153-1)
- Tween-20® (Fisher BP337-100)
- Xylenes, histological grade (Fischer Scientific #X3P1GAL)

EQUIPMENT

- 65°C oven
- Fume hood
- Analytical balance
- Decloaking Chamber™ Pro, (Biocare Medical, Concord, CA)

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REAGENT SETUP

Phosphate Buffered Saline (PBS, 1L of a 1X working solution)

- Dissolve one packet of Dulbecco's Phosphate Buffered Saline in 1L of H_2O and sterilize by autoclaving. Store at 4°C.

Tris Buffered Saline-Tween-20 (TBSTw, 500 mL of a 10X stock solution)

- Combine 15.76g Tris-HCl, 43.83g NaCL, 500 mL H_2O, and 5 mL Tween-20 and adjust pH to 7.4. Store at 4°C.

10% (wt/vol) Roche Reagent (100 mL of a 10X stock solution)

stock solution	final conc.	for 100 mL
maleic acid	100 mM	1.2 g
5 M NaCl	150 mM	3 mL
dH_2O to vol.	--	to 100 mL
Blocking reagent	10%	10 g

- Mix maleic acid, NaCl & dH_2O according to above, pH to 7.5 (note: this is a strong buffer and it is difficult to adjust pH, try using solid NaOH pellets to raise pH initially). Add blocking reagent, microwave briefly to aid solubility. Aliquot 10 mL volumes into conical tubes & store at –20°C for up to three years.

> **CAUTION**
>
> Avoid boiling over, solution will be cloudy & viscous so watch carefully to ensure blocking reagent is completely dissolved.

Citrate Buffer (100x stock 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 Solution (100 mL)

- Combine 10 mL of 10% Roche reagent, 5 mL donkey serum, 1 g BSA Fraction V, and 85 mL TBSTw. Prepare 1 mL aliquots and store at -20°C for up to three years.

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Sudan Black B (50 mL)

- Make a 0.1% stock solution of sudan black by dissolving 50 µg of powder into 50 mL of 70% ethanol.
- Store solution at room temperature
- Make fresh prior to each staining procedure

> **CAUTION**
>
> Use only in area provided with appropriate exhaust ventilation.

Mounting Medium (10 mL)

- Make a n-propyl gallate 20% stock solution by combining 0.4 grams n-propyl gallate and 2 ml DMSO, prepare 100 microliter aliquots and store at -20°C
- Make mounting medium by combining 0.5 mL of 20X PBS solution, 9 mL glycerol, 100 microliters of 20% n-propyl gallate, and 0.5 mL H_2O. Store solution at 4°C for up to 2 months.

4',6-diamidino-2-phenylindole, dilactate (DAPI) solution (10 mL of a 300 µM/ 10X stock solution).

- Combine 0.0014 g DAPI and 10 mL N,N Dimethylformamide. Prepare 100 µl aliquots and store in dark at -20°C for up to 1 year.

Procedure

Dewaxing, antigen retrieval and blocking.

Timing: ~4 h

1. Heat slides to 65°C for 5 min to remove wrinkles and increase tissue adhesion.
2. Dewax and rehydrate slides by incubating at 25°C in Xylene (3 min, repeat twice), 100% ethanol (3 min, repeat twice), 75% ethanol (3 min, repeat twice) and 50% ethanol (3 min, repeat twice).
3. Prepare a 1X working solution of citrate buffer by adding 5 mL of 100X solution to 495 mL of dH20 and place into a 8”x 8”x 2” Pyrex glass dish.
4. Place slides in glass dish and microwave on power 50 for 20 min. Allow slides to cool to room temperature before proceeding.

> **TROUBLESHOOTING**

5. Use a kimwipe to create a dry rectangle around the tissue section.
6. Outline samples with hydrophobic pen, being careful to mark only the dry part of the slide.

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7. Pipette a bead of TBSTw into the outlined region and let sit for 5 min at 25°C with no agitation so that the pap barrier can dry.

> **CRITICAL STEP**
>
> Incomplete drying of hydrophobic barrier will cause tissue sections to dehydrate and will increase background staining

8. Tap off TBSTw and block for 1 hr at 25°C with gentle agitation in blocking buffer.

> **PAUSE POINT**
>
> Samples can be maintained for up to 4 hours

9. Dilute primary antibody/antibodies in blocking buffer, apply to slides, and incubate overnight at 4°C with gentle agitation.

Secondary antibody and coverslipping.

Timing: ~4 h

10. Wash 5 min in TBSTw at 25°C with gentle agitation (repeat 5 times).
11. Dilute flurophore-conjugated secondary antibody/antibodies in blocking buffer, apply to slides, and incubate for 60 min at 25°C in a light protected box.
12. Remove secondary antibody solution and wash with TBSTw for 5 min at 25°C in light protected box. Repeat this step 7 times.

> **TROUBLESHOOTING**

13. Prepare DAPI working solution by combining 1 µl DAPI stock with 999 µl TBSTw, apply to slides and incubate for 5 min at 25°C in light protected box.

14. Wash with TBSTw for 5 min at 25°C in light protected box. Repeat this step 3 times.

15. Add a small bead of antifade-mounting media to slide and add cover glass (Corning #2865-18). Image ASAP.

> **PAUSE POINT**
>
> Samples can be refrigerated (4°C) for up to 3 days before imaging, without appreciable loss of fluorescence.

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Table 1: Troubleshooting table.

Step	Problem / Alternative goal	Possible Reason	Solution
4	Antigen not detected	Ineffective antigen retrieval	To aid in Edu/BrdU retrieval, incubate in 2N HCl at 25°C for 30 min prior to antigen retrieval. Antigien retrieval can also be performed in the decloaker by immersing slides in an alkaline solution (10 mM Tris Base, pH 9.5) or a neutral solution (10 mM Tris HCl, pH 7.5) and running a two incubation cycle (cycle 1: 125°C for 5 min; cycle 2: 72°C for 0.5 min) or on the benchtop by trypsin digestion (phosphate buffered saline containing 0.1% trypsin, incubate at 37°C for 30 min) or proteinase K digestion (phosphate buffered saline containing 5 µg/mL proteinase K solution, incubate at 25°C for 5 min).
11	Cell membrane counterstaining needed		Add TBSTw containing Wheat Germ Agglutinin Texas Red (1:200 dilution from 1mg/ml glycerol stock, Invitrogen #W21405). Incubate for 5 min at 25°C in light protected box and tip off to remove and wash with TBSTw twice for 5 min per wash
11	High fluorescent background staining		Add 0.1% Sudan Black B solution directly onto tissue section. Incubate for 25 min at 25°C. To remove excess solution, wash with TBSTw 3 times for 5 min per wash.

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