University of Michigan Comprehensive Cancer Center	SOP-BCR-5.11	**Doubling Staining for paraffin sections (Factor VIII (NeomMarkers), ALDH1 mouse primary antibody (BD transduction lab)**	Author: S. Clouthier Approved: M. Wicha	Rev: 1.0	Issued: 09/24/98 Revised: 7/17/12

1.0 Purpose

The purpose of SOP 5.11 is to provide instructions for doubling staining for paraffin sections (factor VIII NeoMarkers), ALDH1 mouse primary antibody (BD transduction lab)

2.0 Scope

SOP 5.11 is intended to cover all resources, personnel and equipment in the BCR laboratory.

3.0 Materials

	Name	Description	Storage Location
1.0	Peroxidase quenching solution	(3% hydrogen peroxidase in methanol)	
2.0	1x PBS	Phosphate Buffering Saline	Cold Storage (026-380C)
3.0	Serum Blocking Solution	Reagent A in DAB/AEC kit	Fridge #1 (026-328S-A)
4.0	Biotinylated second antibody	Reagent B in DAB/AEC kit	Fridge #1 (026-328S-A)
5.0	Enzyme conjugate	Reagent C in DAB/AEC kit	Fridge #1 (026-328S-A)
6.0	AEC Single Solution	Reagent D in AEC Kit	Fridge #1 (026-328S-A)

4.0 Procedure

4.1 Deparaffine the slides cut from paraffin-embedded Tissue sections.

- 3x xylenes for 10 minutes each.
- 2x 100% EtOH for 5 minutes each.
- Wash in dH_20 for 5 minutes on a rocker plate.
- When conducting Step 1, please set the water bath fo 98° C

4.2 Antigen unmasking (Retrieve): Soak the slides in heated (98° C water bath) ready-to-use Citrate Buffer (pH 6.0) for 40 minutes. Take the slides out together with the buffer from the water bath and let it cool down to room temperature (takes more than 20 minutes). Wash in dH_20 for 15 minutes.

4.3 Submerge slides in Peroxidase quenching solution (cold 3% hydrogen peroxide in methanol) for 10 minutes.

4.4 Wash in 1x PBS for 15 minutes with several changes of PBS.

4.5 **DAB Kits and AEC can share the same reagents except for the Chromagen!**

4.6 **Start to use the Histostain®-*Plus* kits (DAB kits) for Factor VIII staining.**

4.7 Add enough Serum blocking solution (reagent A) to completely cover tissue. Incubate for 10 minutes. Drain or blot off solution. **Do not rinse!**

4.8 PRIMARY ANTIBODY

- Tap off excess Serum blocking solution and wipe slides as before. Apply enough optimally diluted primary antibody (1:100 dilution of Factor VIII Primary Antibody in 1x PBS) to cover cells. Incubate 1 hour at room temperature.
- Wash in fresh 1x PBS for > 15 minutes with several changes of 1x PBS.

4.9 Apply enough Biotinylated Second Antibody (Reagent B) to completely cover the cells. Incubate for 10 minutes.

4.10 Wash in 1x PBS for 15 minutes with several changes of PBS.
4.11 Apply enough Enzyme Conjugate (Reagent C) to completely cover the cells. Incubate for 10 minutes.
4.12 Wash in 1x PBS for 15 minutes with several changes of PBS.
4.13 Add 1 drop of reagent 3A, 1 drop of reagent 3B, and 1 drop of reagent 3C to 1 mL distilled water, mix well and protect from light. Incubate slides at room temperature for 12 minutes (need to observe under microscope).
4.14 Rinse well with distilled water for five minutes and then in 1x PBS for ten minutes.
4.15 **Start to use the Histostain®-*Plus* Kits (AEC kit from Zymed Lab, Cat. 85-9943) for GFP staining**
4.16 Add enough Serum Blocking Solution (Reagent A) to completely cover tissue. Incubate for 10 minutes. Then drain or blot off solution. **Do not rinse!**
4.17 PRIMARY ANTIBODY

- **Don't let ALDH get warm!**
- Tap off excess Serum blocking solution and wipe slides as before. Apply enough optimally diluted primary antibody (1:50 dilution of ALDH1 primary antibody in 1x PBS) to cover cells. Incubate 1 hour at room temperature.
- Wash in fresh 1x PBS for > 15 minutes with several changes of 1x PBS.

4.18 Apply enough enzyme conjugate (Reagent C) to completely cover the cells. Incubate for 10 minutes.
4.19 Wash in 1x PBS for 15 2 changes of PBS.
4.20 DO NOT EQUILIBRATE AEC *Single Solution* TO ROOM TEMPERATURE BEFORE USE! Add cold (2-8°C) AEC Single Solution (Reagent D) to slides. Immediately return AEC *Single Solution* back to 2-8° C storage. Incubate slides at room temperature for 1-2 minutes (need to observe under microscope).
4.21 Rinse well with distilled water.
4.22 HEMATOXYLIN COUNTERSTAIN (optional)

- Apply enough hematoxylin to cover specimen. Length of incubation depends on the strength of hematoxylin used (1 minute).
- Rinse gently in a distilled water bath for > 15 minutes.
- Check the color under microscope.

4.23 MOUNTING

- Specimens may be mounted and cover slipped with an aqueous-based mounting medium such as Glycerin (don't dehydrate the cells)
- Slides may be stored up to one year.

5.0 Applicable References

6.0 Change Description

Revision	Date	Reference	Description of Change
1.0	7/17/12	CL	Added room locations