University of Michigan Comprehensive Cancer Center	SOP-BCR-5.14	**Doubling Staining for Paraffin section (ERa rabbit primary antibody (Neomarker Rb-9016-PO), GFP mouse monoclonal primary antibody (MS-1288-p, Neomarker))**	Author: S. Clouthier Approved: M. Wicha	Rev: 1.0	Issued: 09/24/98 Revised: 7/17/12

1.0 Purpose

The purpose of SOP 5.14 is to provide instructions on how to stain for Paraffin sections (ERa rabbit primary antibody and GFP mouse monoclonal primary antibody (MS-1288-p) Neomarker)

2.0 Scope

SOP 5.14 is intended to cover all resources, personnel and equipment in the BCR laboratory.

3.0 Materials

No.	Name	Description	Storage Location
1.0	1x PBS	Phosphate buffering saline	Cold Storage (026-380C)
2.0	Citrate Buffer	For immunohistochemical staining	Fridge 1/Cold Storage (026-328S-A/026-380C)
3.0	Serum blocking solution	Reagent 1A in DAB kit	Fridge #1 (026-328S-A)
4.0	Biotinylated Second antibody	Reagent 1B in DAB kit	Fridge #1 (026-328S-A)
5.0	Enzyme conjugate	Reagent 2 in DAB kit	Fridge #1 (026-328S-A)
6.0	ERa	Primary Antibody (1:100 dilution of ERa primary antibody in 1xPBS)	Fridge #1 (026-328S-A)
7.0	Serum Blocking Solution	Reagent A in AEC kit	Fridge #1 (026-328S-A)
8.0	Primary Antibody	1:50 dilution of GFP primary antibody in 1x PBS)	Fridge #1 (026-328S-A)
9.0	Biotinylated Second antibody	Reagent B in AEC kit	Fridge #1 (026-328S-A)
10.0	Enzyme conjugate	Reagent C in AEC kit	Fridge #1 (026-328S-A)
11.0	AEC Single Solution		Door of Fridge #2, brown bottle (026-328S-A)
12.0	Hematoxylin	Staining agent	

4.0 Procedure

4.1 Always shake everything before using!
4.2 Glass dish needs to be moist, wet paper towel

4.3 Deparaffine the slides cut from paraffin-embedded Tissue sections
- 3x xylenes for > 15 minutes each
- 2x 100% EtOH for 5 minutes each
- Wash in d H20 for > 5 minutes in running water.

4.4 Antigen unmasking (Retrieve): Soak the slides in ready to use Citrate Buffer (PH 6.0) and heated at 98° C for 40 minutes and let it cool down for about 10 minutes (room temperature). Wash in dH20 for 3-4 minutes.

4.5 Submerge slides in Peroxidase quenching solution for 10 minutes.

4.6 Wash in PBS for 15' with several changes of PBS.

4.7 Apply blocking serum as mentioned.
- **Start to use the Histostain®-Plus Kits (DAB kit from Zymed Lab, Cat. 95-9643) for ERa staining.**

4.8 Add enough Serum Blocking Solution (Reagent 1A) to completely cover tissue. Incubate for 10'. Then, drain or blot off solution. **DO NOT RINSE.**

4.9 PRIMARY ANTIBODY. Tap off excess Serum blocking solution and wipe slides as before. Apply enough optimally diluted primary antibody (**1:100** dilution of **ERa** primary antibody in 1x PBS) to cover cells. Incubate 1 hour at RT. Wash in fresh PBS for > 10 minutes with > 2 changes of 1x PBS.

4.10 Apply enough biotinylated second antibody (Reagent 1B) to completely cover the cells. Incubate for 10 minutes.

4.11 Wash in fresh PBS for >10 minutes for > 2 changes of PBS.

4.12 Apply enough enzyme conjugate (Reagent 2) to completely cover the cells. Incubate for 10'.

4.13 Wash in fresh PBS for >10 minutes and >2 changes of PBS.

4.14 Add one 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 3-5 (7) minutes (**need to observe under microscope**)

4.15 Rinse well with distilled water and then in PBS.

4.16 **Start to use the Histostain®-Plus Kits (AEC kit from Zymed Lab, Cat. 85-9943) for GFP staining.**

4.17 Add enough serum blocking solution (Reagent A) to completely cover tissue. Incubate for 10' then drain or blot off solution. DO NOT RINSE.

4.18 PRIMARY ANTIBODY: Tap off excess serum blocking solution and wipe slides as before. Apply enough (250 µL) optimally diluted primary antibody (1:50 dilution of GFP primary antibody in PBS) to cover cells. Incubate 1 hour at RT. Wash in fresh PBS for > 15 minutes with several changes of PBS.

4.19 Apply enough Biotinylated Second Antibody (reagent B) to completely cover the cell. Incubate for 10 minutes.

4.20 Wash in PBS for 15 minutes with several changes of PBS.

4.21 Apply enough enzyme conjugate (Reagent C) to completely cover the cells. Incubate for ten minutes.

4.22 Wash in PBS for 15 minutes with several changes of PBS.

4.23 DO NOT EQUILIBRATE AEC Single Solution to ROOM TEMPERATURE BEFORE USE! Add cold (2-8°C) AEC Single Solution to slides. Immediately return AEC Single Solution back to 2-8°C storage. Incubate slides at room temperature for 10-15 minutes. You need to observe under the microscope.

4.24 Rinse well with distilled water.
4.25 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.
4.26 Check the color under the microscope.
4.27 MOUNTING: Specimens may be mounted and cover slipped with an aqueous-based mounting medium such as Glycerin (don't dehydrate the slides).
4.28 You may store the slide 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