1.0 Purpose
The purpose of SOP 5.2 is to provide instructions for doubling staining for paraffin sections using GFP mouse primary antibody.

2.0 Scope
SOP 5.2 is intended to cover all resources, personnel and equipment in the BCR laboratory.

3.0 Materials

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Description</th>
<th>Storage Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>Peroxidase quenching solution</td>
<td>3% Hydrogen Peroxidase in Methanol</td>
<td>Cold Storage (026-380C)</td>
</tr>
<tr>
<td>2.0</td>
<td>1x PBS</td>
<td>Phosphate Buffering Saline</td>
<td>Fridge #1</td>
</tr>
<tr>
<td>3.0</td>
<td>Serum Blocking Solution</td>
<td>Reagent 1A in DAB kit, Reagent A in AEC</td>
<td>Fridge #1</td>
</tr>
<tr>
<td>4.0</td>
<td>Biotinylated second antibody</td>
<td>Reagent 1B in DAB/, Reagent B in AEC kit</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>Enzyme conjugate</td>
<td>Reagent 2 in DAB kit, Reagent C in AEC kit</td>
<td></td>
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<tr>
<td>6.0</td>
<td>AEC Single Solution</td>
<td>Reagent D in AEC Kit</td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>GFP</td>
<td>Green Flourescent Protein</td>
<td>Freezer #2</td>
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</tbody>
</table>

4.0 Procedure

4.1 Deparaffine the slides cut from paraffin-embedded tissue sections.
- 3x xylene for 10 minutes each.
- 2x 100% EtOH for 5 minutes each.
- Wash in dH2O for 5 minutes on a rocker plate.
- When conducting Step 1, please set the water bath to 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 dH2O 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
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 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 12 minutes (need to observe under microscope).

4.14 Rinse well with distilled water.

4.15 HEMATOXYLIN COUNTERSTAIN (optional)

4.16 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

<table>
<thead>
<tr>
<th>Revision</th>
<th>Date</th>
<th>Reference</th>
<th>Description of Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>7/17/12</td>
<td>CL</td>
<td>Added room locations</td>
</tr>
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