Paraffin Sections Immunohistochemistry Protocol  
for mouse macrophage marker F480 and B cell marker B220  
on control Mouse Spleen  

Date:  

Materials:  
1. Bovine Serum Albumin: Sigma Catalog A4503  
2. Hydrogen Peroxide: Fisher Catalog H325-100  
3. Avidin Biotin Blocking Kit Vector labs Catalog SP2001 or Biolegend Catalog 927301  
4. Heat induce Antigen Retrieval Citrate buffer pH 6.0 for F480 may be needed  
5. Proteinase K Dako Cat# S3020 pre-diluted ready to use  
7. Biotinylated B220 Catalog: BD 553086  
8. HRP Streptavidin Catalog: Jackson Immunoresearch 016-030-084  
9. AEC Substrate: Vector Catalog SK4200  
10. Mayer’s Hematoxylin Sigma MHS32  
11. Aqueous mounting media Aquamount Vector labs catalog H5501  
12. Washing Buffer: Phosphate Buffered Saline with 0.1%Tween20 (PBST)  
13. Diluting Buffer: 1% Bovine Serum Albumin in PBST (BSA/PBST)  

Protocol  

1. De-Paraffinize Slides:  
   __ 3x Xylene for 10 min each (30 minutes total)  
   __ 2x 100% EtOH for 5 min each (10 minutes total)  
   __ 2x 95% EtOH for 5 min each (10 minutes total)  
   __ 2x 70% EtOH for 5 min each (10 minutes total) followed by 3x Wash in PBST  

2. Blocking Steps:  
   a. Block Endogenous Peroxidase (if using peroxidase substrate)  
      __ 0.3% H₂O₂ in Wash Buffer for 30 minutes followed by 3x Wash in PBST  
   b. Block Endogenous Biotin (if using biotinylated antibodies)  
      __ Apply Avidin from blocking kit for 15 minutes followed by 3x Wash in PBST  
      __ Apply Biotin from blocking kit for 15 minutes, followed by 3x Wash in PBST  

3. Antigen Retrieval:  
   a. Heat-Induced Epitope Retrieval (HIER)  
      __ Place slides in complete rack, use dummy slides as necessary  
      __ Heat slides in a Citrate Buffer w/ water bath for 5 minutes  
      __ Check slides and then repeat for 5 minutes  
      __ Allow cool down at room temp for exactly 20 minutes  
   OR  
   b. Enzyme-Induced Epitope Retrieval  
      __ Proteinase K for 6 minutes followed by 3x Wash in PBST  

4. Block Hydrophobic Interactions, if necessary before adding diluted primaries  

5. Primary Application: overlay about 200 microliters of diluted primary reagent to each appropriately labeled slide and if incubating overnight cover each slide with parafilm to prevent drying out, even if incubating in a humid chamber. The next morning remove the parafilm and wash slides 3x in PBST  

6. Secondary Streptavidin application: to detect binding of biotinylated primary, overlay with appropriately diluted secondary for 30 minutes in a humid chamber followed by 3x Washes in PBST
7. **Substrate overlay to detect binding:** Make AEC following manufacturer’s instructions. Test a drop on a few microliters of secondary and overlay onto slides and cover to let reaction occur—for 5-20 minutes.

8. Stop reaction if negative control shows any color and wash slides 3x in PBST. The positive control should turn color quickly.

9. **Counterstain nuclei** in Aqueous Mayer’s Hematoxylin for 2 minutes and wash slides in distilled water and let slides dry on the bench.

10. **Coverslip** in Aqueous mounting media (AEC IS Alcohol soluble unlike DAB)

11. View and proceed to digital photography with scale bars.

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<table>
<thead>
<tr>
<th>#</th>
<th>Primary Reagents</th>
<th>Catalog Number</th>
<th>Dilutions</th>
<th>Incubation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>BSA/PBST</td>
<td></td>
<td>1:100</td>
<td>Overnight @ 4°C</td>
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<tr>
<td>2.</td>
<td>biotinylated F4/80</td>
<td>MCA497BB Lot: 1603</td>
<td>1:50</td>
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<td>3.</td>
<td>biotinylated rat anti-B220</td>
<td>BD 553086 Lot: 49369</td>
<td>1:100</td>
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</tbody>
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<table>
<thead>
<tr>
<th>#</th>
<th>Secondary Reagents</th>
<th>Slides Applied To</th>
<th>Dilutions</th>
<th>Incubation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>HRP StreptAvidin 016-030-084 Lot 135331</td>
<td>ALL</td>
<td>1:500</td>
<td>30 min @ RT</td>
</tr>
</tbody>
</table>

**Substrate Application**

- a. Reagent: AEC apply for ____ minutes
- 3x Wash Buffer

**Counterstain:** Mayer’s Hematoxylin for 2 minutes.

3x Wash Buffer

**Air Dry Slides:** __

**Cover Slip in proper medium (Vectamount, white bottle): __

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Examples of results on mouse spleen

- **mouse spleen with negative control**
- **mouse spleen with anti-B220 B cell marker**
- **mouse spleen with anti-F480 anti-macrophage**