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

Date: **Samples/# of slides:**

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
6. Biotinylated F480 BioRad (Serotec) Catalog: MCA 497BB
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% H_2O_2 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 dtect binding of biotinylted 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 instrucitons. 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

#	Primary Reagents	Catalog Number	Dilutions	Incubation Time
1.	BSA/PBST		1:100	**Overnight @ 4°C**
2.	*biotinylated* F4/80	MCA497BB Lot: 1603	1:50	↓
3.	*biotinylated* rat anti-B220	BD 553086 Lot: 49369	1:100	

__ 3x Wash Buffer

#	Secondary Reagents	Slides Applied To	Dilutions	Incubation Time
1.	HRP StreptAvidin 016-030-084 Lot 135331	ALL	1:500	**30 min @ RT**

__ 3x Wash Buffer

Substrate Application __

a. Reagent: AEC apply for __**minutes**__
   3x Wash Buffer

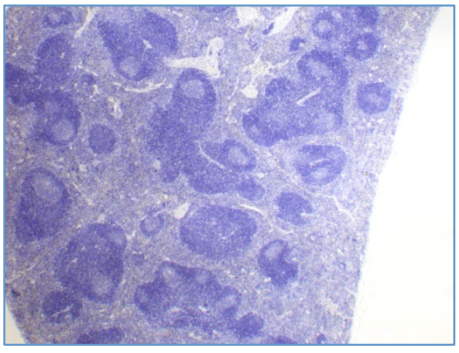
HRP Test_

Counterstain: Mayer's Hematoxylin for **2 minutes**_ 3x Wash Buffer

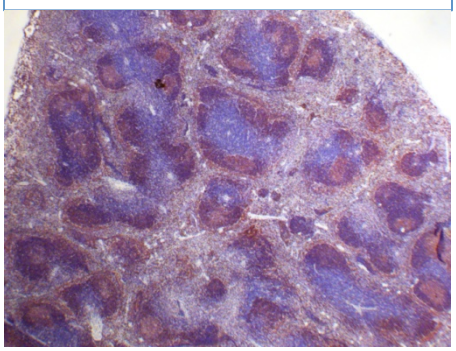
Air Dry Slides: __

12. Coverslip in proper medium (Vectamount, white bottle): __

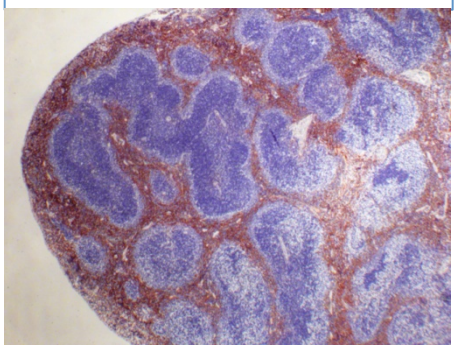
mouse spleen with negative control



mouse spleen with anti-B220 B cell marker



mouse spleen with anti-F480 anti-macrophage



Examples of results on mouse spleen