

### DOUBLE STAINING OF SPLLEN SECTIONS

Throughout this procedure do not use any solutions that contain azide.

All dilutions are made with PBS/5% BSA + 0.1% Tween 20.

All washes are in PBS/ 5% BSA/ 0.1% Tween 20.

1. Allow the sections to warm up to room temperature.
2. Apply PAP pen (Research Products) around section and allow to dry. This prevents Ab dilutions from floating all over the slide.
3. Place slides in PBS for 20 minutes.
4. Place slides in 0.3% H<sub>2</sub>O<sub>2</sub> (diluted in water) for 5 minutes to destroy endogenous peroxidase activity.
5. Place slides in PBS for 20 minutes.
6. Incubate the sections in PBS/ 5% BSA/ 0.1% Tween 20 for 20 minutes.
7. Pour off and add 100  $\mu$ l of biotinylated antibody and incubate at room temperature in a humidified chamber for 60 minutes.
8. Wash three times in PBS/BSA/Tween 20 with shaking 10 minutes each wash.
9. Add 100  $\mu$ l Streptavidin-AP (Southern Biotechnology; 1:400 dilution) and incubate at room temperature in chamber for 45 minutes.
10. Wash three times in PBS/BSA/Tween 20 with shaking, 10 minutes each wash.
11. Add 100  $\mu$ l HRP conjugate (usually PNA-HRP) and incubate at room temperature in chamber for 60 minutes.
12. Wash three times in PBS/BSA/Tween 20 with shaking, 10 minutes each wash.
13. Wash in PBS for 10 minutes.
14. Develop Alk Phos first with Fast BB Blue and Napthol AS-MX-phosphate followed by 3-aminoethylcarbazole (3-AEC) for HRP. Refer to protocol for preparation of these two substrates. In double staining do not use NBT/BCIP for Alk Phos because the blue/black color produced does not contrast well with the red HRP reaction product with 3-AEC.

Fast Blue BB/Naphthol AS-MX Phosphate yields a sky blue product while 3-AEC gives a crimson red product. Both these reaction products are water insoluble but soluble in EtOH. Do not dehydrate in graded ethanols.

15. Mount using Biomeda Crystal Mount (Fisher Scientific). Add 50  $\mu$ l Crystal Mount directly on top of the wet section; allow to remain at room temperature for 5 mins; place slides in 70° C oven to harden (about 10 mins). Now add a few drops of Permunt and a coverslip.