

IMMUNOHISTOCHEMISTRY

For Use with Fixed and Frozen or Decellodized Tissue

<p>1. Wash 2x 5 mins in PBS [Phosphate Buffered Saline].</p> <p><i>Use ~8-10 mL per beaker</i></p> <p><i>Omit steps 2 & 3 for immunofluorescence (IF)</i></p>	<p>1. PBS is a formulation of pH stabilizers and salts designed to wash away unbound components without suppressing antigen-antibody binding interactions, reducing nonspecific background and increasing the specific signal while maintaining the proper buffering environment. Five minutes is minimum wash time (longer is not deleterious).</p> <p><i>Peroxidase blocking only necessary for DAB staining.</i></p>
<p>2. Incubate tissue for 20 mins in H₂O₂ [Hydrogen Peroxide] solution.</p> <p><i>For every 1000μL of PBS, add 20μL H₂O₂ & 10μL MeOH.</i></p>	<p>2. Diluted H₂O₂ is used to reduce nonspecific background staining due to endogenous peroxidase. Pre-treatment with saturating amounts of hydrogen peroxide results in the irreversible inactivation of endogenous peroxidase.</p>
<p>3. Repeat washes from Step 1</p>	<p>3. See #1</p>
<p>3a. For anti-BrdU staining:</p> <p>a. Incubate tissue in HCl (0.5N) for 6min @ 55°C (water bath).</p> <p><i>For every ~917μL of PBS, use ~83.3μL of 6N HCl stock (can simplify to 900:100 ratio).</i></p> <p>b. PBS washes - 3x5 min @ RT</p> <p>c. Incubate in Proteinase K (5μg/mL) in TE buffer for 2 min @ 37°C (water bath).</p> <p><i>For every 1000μL TE buffer, use 0.25μg/mL stock Proteinase K solution.</i></p> <p>d. PBS washes - 3x5 min @ RT</p>	<p>4. For anti-BrdU staining:</p> <p>a - d. Aids in breaking apart the nuclear membrane for proper staining.</p>

<p>3b. For anti-WGA staining:</p> <ol style="list-style-type: none"> a. Add 0.04g Acetone Rat Brain Powder and 2μL anti-WGA antibody to 1mL PBS. Incubate at room temperature on shaker table for 1 hour. b. Centrifuge @ 13,000rpm for 10 min. c. Remove supernatant, repeat b. in fresh centrifuge tube. d. Add supernatant to beaker of PBS at 1:5000 final dilution along with 5% Normal serum. <p><i>For 10mL final volume, use 1mL of antibody solution, and 9mL PBS</i></p>	<p>5. For anti-WGA staining: Rat Brain Powder is used to pre-absorb antibody to reduce background. Any antibody that would bind to rat tissue should be taken up by the powder, and therefore won't stain when applied to the sections. Powder concentration can be varied according to need.</p>
<p>3c. Permeabilize and block tissue in 0.2% Triton-X and Normal Serum (NS) for 1hr @ RT.</p> <p><i>For every 1000μL PBS, add 20μL 10% Triton-X & 25μL NS.</i></p>	<p>6. Triton-X is a detergent that helps break apart the cell membrane to allow antibodies to enter. Normal Serum should come from the host animal that the 2^o antibody was raised in. For example, if the 2^o is biotinylated <u>goat</u> anti-rabbit IgG, Normal <u>Goat</u> Serum should be used in this step. Blocking with Normal Goat Serum ensures that no cross-reactivity with goat antigens occurs.</p>
<p>4. Incubate tissue in 1^o antibody overnight.</p>	<p>4. Incubate overnight to increase binding of primary antibody to its epitope. All antibody is diluted in Triton-X and goat serum to dilute the antisera and aid in antibody penetration of the tissue section.</p>
<p>5. Repeat washes from Step 1</p>	<p>5. See #1</p>
<p>6. Incubate in 2^o antibody 2 hours</p>	<p>6. Secondary antibody will bind to the primary antibody and it is biotinylated (conjugated with biotin) so it will bind the ABC complex as well (see step #8).</p>
<p>7. Repeat washes from Step 1</p>	<p>7. See #1</p>
<p>8. Incubate in ABC [Avidin and Biotinylated horseradish peroxidase macro molecular complex] for 2 hours (make 30 mins in advance)</p>	<p>8. The ABC complex creates a 3-D array containing many biotinylated horseradish peroxidase molecules cross-linked by avidin. Avidin is a glycoprotein with an extremely high affinity for biotin, creating an essentially irreversible binding to the 1^o/2^o antibody complex</p>

9. Repeat washes from Step 1	9. See #1
10. Develop in diaminobenzidine [DAB] solution under hood (250µL DAB, 15µL H ₂ O ₂ , 50mL Tris)	11. DAB is an enzyme mediated antibody detector – it shows peroxidase activity (occurring due to ABC)
11. Rinse in dH ₂ O	12. See #10
12. Place tissue in mounting solution (1:1 ratio of PBS and Subbing Solution)	13. Mounting solution for coverslipping
13. Transfer tissue to subbing solution	14. Place both slide and tissue in small Pyrex dish filled with subbing solution to cover at least half of the slide, use solution to help move tissue onto slides
14. Mount carefully onto subbed slides	15. This is the most important step for preservation of tissue integrity. It will seem tedious at first, but learning different techniques will make the process more efficient.
15. Dry on slide warmer or countertop for at least a few hours.	16. Tissue must be dried onto slide in order to counterstain