Immunohistochemistry (IHC-P) Protocol

Immunohistochemistry (IHC) is a method for demonstrating the presence and location of proteins in tissue sections. Immunohistochemical staining is accomplished to recognize the target protein with antibody which specifically binds to the protein of interest in the tissue section. IHC-P refers to the staining of tissues that have been fixed (usually in neutral buffered formalin) and then embedded in paraffin before being sectioned.

1. Reagents
   - **Xylene**
     Store at room temperature

   - **100% Ethanol**
     Store at room temperature

   - **95% Ethanol**
     95 ml  100% Ethanol
     5 ml   ddH$_2$O
     Store at room temperature

   - **90% Ethanol**
     90 ml  100% Ethanol
     10 ml  ddH$_2$O
     Store at room temperature

   - **80% Ethanol**
     80 ml  100% Ethanol
     20 ml  ddH$_2$O
     Store at room temperature

   - **70% Ethanol**
     70 ml  100% Ethanol
     30 ml  ddH$_2$O
     Store at room temperature
Sodium Citrate Buffer
2.94 g  Sodium citrate trisodium salt dihydrate (C₆H₅Na₃O₇•2H₂O)
0.44g  C₆H₈O₇•2H₂O
1000 ml  ddH₂O
Adjust to pH 6.0
Store at room temperature

Hydrogen Peroxide Buffer
10 ml  30%H₂O₂
90 ml  ddH₂O
Store at room temperature

PBS Buffer
8.5 g  NaCl
1.4 g  Na₂HPO₄
0.2 g  NaH₂PO₄
1000 ml  ddH₂O
Adjust to pH 7.4
Store at 4 °C

Washing Buffer
0.5 ml       Tween 20
1000 ml  PBS Buffer
Store at 4 °C

Antibody Dilution Buffer
3 g   BSA
100 ml  PBS Buffer
Store at 4 °C

Normal Blocking Solution
4.5 ml  PBS Buffer
0.5 ml   Normal goat serum
Store at 4 °C
**Avidin/Biotin Blocking Solution**

2 ml   Egg white
8 ml   PBS Buffer

Store at 4 °C

**DAB Reagent**

**Hematoxylin Reagent**

2. **Procedure**

2.1 Deparaffinization/Rehydration

a. Immerse sections twice with *Xylene* for 10 minutes each time.

b. Immerse sections twice with 100% *Ethanol* for 10 minutes each time.

c. Immerse sections once with 95% *Ethanol* for 5 minutes.

d. Immerse sections once with 90% *Ethanol* for 5 minutes.

e. Immerse sections once with 80% *Ethanol* for 5 minutes.

f. Immerse sections once with 70% *Ethanol* for 5 minutes.

g. Immerse sections with *PBS Buffer* for 5 minutes and immerse the sections in ddH₂O until antigen retrieval.

Note: Do not allow sections to dry at any time until ready to perform antigen retrieval. Drying will cause non-specific antibody binding and therefore high background staining.

2.2. Endogenous peroxidase inhibition

a. Immerse sections with *Hydrogen Peroxide Buffer* for 10 minutes at room temperature.

b. Wash sections with ddH₂O twice for 5 minutes each time.

2.3. Antigen retrieval

a. Immerse section in *Sodium Citrate Buffer* then maintain at 100 °C for 10 minutes.

b. Cool section on bench top for 30 minutes.

c. Wash sections with ddH₂O twice for 5 minutes each time.

2.4. Normal blocking

a. Immerse each section with 100-400 µl of *Normal Blocking Solution* for 1 hour at room temperature.

b. Rinse the excess *Normal Blocking Solution* with a gentle stream of *PBS Buffer* from a wash buffer bottle and wash section with *Washing Buffer* for 10 minutes.

2.5 Avidin/Biotin blocking

a. Immerse each section with 100-400 µl of *Avidin/Biotin Blocking Solution* at 37 °C for 30 minutes.

b. Rinse the excess Blocking Buffer with a gentle stream of *PBS Buffer* from a wash buffer bottle and wash section with *Washing Buffer* for 10 minutes.
2.6. **Primary incubation**
   a. Dilute primary antibody and negative control antibody with *Antibody Dilution Buffer* at proper working concentration according to manufacturer’s guidance.
   b. Add 100-400 μl of diluted primary antibody and negative control antibody to each section and incubate at 37 °C for 1 hour or 4 °C for 12 to 24 hours in a closed incubation chamber.
   c. Rinse the excess antibody from the section with a gentle stream of *PBS Buffer* from a wash bottle and immerse sections in *Washing Buffer* twice for 10 minutes at room temperature.
   
   **Note:** The concentration of antibody should be diluted according to manufacturer’s recommendations.

2.7. **Biotin-labeled secondary antibody incubation**
   a. Dilute Biotin-labeled antibody with *Antibody Dilution Buffer* at proper working concentration according to manufacturer’s guidance.
   b. Add 100-400 μl of diluted Biotin-labeled antibody to each section and incubate at 37 °C for 45 minutes in an incubation chamber.
   c. Rinse the excess antibody from the section with a gentle stream of *PBS Buffer* from a wash bottle and immerse sections in *Washing Buffer* twice for 10 minutes at room temperature.

2.8. **Streptavidin-HRP incubation**
   a. Dilute Streptavidin-HRP antibody with *Antibody Dilution Buffer* at proper working concentration according to manufacturer’s guidance.
   b. Add 100-400 μl of diluted Streptavidin-HRP to each section and incubate at 37 °C for 30 minutes in an incubation chamber.
   c. Rinse the excess antibody from the section with a gentle stream of *PBS Buffer* from a wash bottle and immerse sections in *Washing Buffer* twice for 10 minutes at room temperature.

2.9. **DAB stain**
   a. Add 100-400 μl of *DAB Reagent* or other substrate to each section and react for 2 to 7 minutes to stain.
   b. Rinse sections with *PBS buffer* to stop staining.
   
   **Note:** The following step is additional but not necessary.

2.10. **Hematoxylin stain**
   Counterstain sections in *Hematoxylin Reagent* according to manufacturer’s instructions.

2.11. **Dehydrate sections**
   a. Immerse sections with 95% *Ethanol* twice for 10 seconds each time.
   b. Immerse sections with 100% *Ethanol* twice for 10 seconds each time.
   c. Immerse sections with *Xylene*, incubating sections for 10 seconds each time.

2.12. **Mount coverslips**

2.13. **Viewing the staining under the microscope.**
3. Immunohistochemistry examples

Immunohistochemistry analysis of human carcinoma hepatis tissue slide (Paraffin embedded) using Ki-67 Antibody, pAb, Rabbit (Left, GenScript, A01495) and Purified Rabbit IgG (Whole molecule) Control (Right, GenScript, A01008).

Immunohistochemistry analysis of human brain tissue slide (Paraffin embedded) using β-Tubulin III Antibody, pAb, Rabbit (Left, GenScript, A01203) and Purified Rabbit IgG (Whole molecule) Control (Right, GenScript, A01008).

4. Recommended Products

<table>
<thead>
<tr>
<th>Name</th>
<th>Cat. No.</th>
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</thead>
<tbody>
<tr>
<td>Streptavidin-HRP</td>
<td>M00091</td>
</tr>
<tr>
<td>Human IgG Control (Whole Molecule), Purified</td>
<td>A01006</td>
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<tr>
<td>Mouse IgG Control (Whole Molecule), Purified</td>
<td>A01007</td>
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<tr>
<td>Rabbit IgG Control (Whole Molecule), Purified</td>
<td>A01008</td>
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<tr>
<td>Goat IgG Control (Whole Molecule), Purified</td>
<td>A01009</td>
</tr>
<tr>
<td>Chicken IgY Control (Whole Molecule), Purified</td>
<td>A01010</td>
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## 5. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Solution</th>
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</thead>
<tbody>
<tr>
<td>Weak or no staining</td>
<td>Inadequate deparaffinization</td>
<td>Deparaffinize sections longer or change fresh xylene</td>
</tr>
<tr>
<td></td>
<td>Inactive primary antibodies</td>
<td>Replace with a new batch of antibodies</td>
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<tr>
<td></td>
<td>Antibody concentration is too low</td>
<td>Increase the concentration of primary and/or secondary antibodies</td>
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<tr>
<td></td>
<td>Incompatible secondary and primary antibodies</td>
<td>Use secondary antibody that will interact with primary antibody</td>
</tr>
<tr>
<td></td>
<td>Inactive secondary antibody</td>
<td>Replace with a new batch of antibody</td>
</tr>
<tr>
<td>Overstaining</td>
<td>Inadequate substrate incubation time</td>
<td>Increase the substrate incubation time</td>
</tr>
<tr>
<td></td>
<td>The concentration of primary and/or secondary antibodies is too high</td>
<td>Reduce antibody concentration or perform a titration to determine the optimal dilution for primary and secondary antibodies</td>
</tr>
<tr>
<td></td>
<td>Incubation time is too long</td>
<td>Reduce incubation time</td>
</tr>
<tr>
<td></td>
<td>Substrate incubation time is too long</td>
<td>Reduce substrate incubation time</td>
</tr>
<tr>
<td></td>
<td>Sections dried out</td>
<td>Avoid sections being dried out</td>
</tr>
<tr>
<td>High background</td>
<td>Inadequate washing of sections</td>
<td>Wash at least 3 times between steps</td>
</tr>
<tr>
<td></td>
<td>Non-specific binding of primary antibodies to tissue or antibody concentration was too high</td>
<td>Non-specific binding may be reduced by using higher dilution of primary antibodies</td>
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<tr>
<td></td>
<td>Non-specific binding of secondary antibodies to tissue</td>
<td>Treat tissue with normal serum from the same species as secondary antibodies.</td>
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<tr>
<td></td>
<td>Secondary antibodies cross react with similar species of tissue</td>
<td>Use pre-adsorbed 2nd antibody, i.e. use rabbit anti-rat IgG, mouse adsorbed, on mouse tissue, or use rabbit anti-mouse IgG, rat adsorbed, on rat tissue</td>
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<tr>
<td></td>
<td>Diffusion of tissue antigen due to inadequate fixation</td>
<td>Increase duration of postfixation</td>
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