Vector avidin/biotin reagents and ABC systems are carefully produced to ensure consistent and optimal staining, rigorously tested using a variety of immunohistochemical applications, and guaranteed to retain activity during prolonged storage. However, background staining may be present, or specific staining may be absent or diminished in test specimens due to factors intrinsic to the tissue or cell samples (i.e. fixation, antigen expression, endogenous tissue components, etc.). Not all of the causes of off-target staining or weak staining may be obvious. Trying to solve the problem often becomes a time-consuming and frustrating task. We hope this troubleshooting guide helps to identify and correct the most common sources of problems encountered in IHC/ICC staining.

If your problem is not solved using this guide, please contact our technical service staff for further assistance (by phone: (650) 697-3600 or email: vector@vectorlabs.com).

**TROUBLESHOOTING GUIDE**

## Avidin-Biotin (ABC) Systems

To evaluate background staining, run these deletion controls.

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td><img src="image" alt="A" /></td>
<td><img src="image" alt="B" /></td>
<td><img src="image" alt="C" /></td>
<td><img src="image" alt="D" /></td>
<td><img src="image" alt="E" /></td>
</tr>
<tr>
<td>Substrate</td>
<td>ABC reagent may bind to tissue samples for several reasons:</td>
<td>Secondary antibody can bind to endogenous immunoglobulins if used to detect a primary antibody that is of the same species as the tissue (e.g. mouse primary on mouse tissue).</td>
<td>- Blocking Serum</td>
<td>Excess primary antibody has been used.</td>
</tr>
<tr>
<td>Staining</td>
<td>• Endogenous protein-bound biotin</td>
<td>For mouse primary on mouse tissue, use M.O.M.™ Kit (PK-2200, MP-2400, or BMK-2202)</td>
<td>- Biotin</td>
<td>Titer the primary antibody concentration.</td>
</tr>
<tr>
<td><img src="image" alt="A" /></td>
<td>• Endogenous lectin</td>
<td>Cross-reactivity may occur between secondary antibody and tissues that are related in species to primary antibody or have tissue components with related amino acid sequences to the primary.</td>
<td>- Secondary Antibody</td>
<td>The optimal antibody concentration should produce clean specific staining with no background.</td>
</tr>
<tr>
<td><img src="image" alt="A" /></td>
<td>• Ionic interactions</td>
<td>• Use secondary antibodies that have been adsorbed for the tissue species.</td>
<td>- Biotinylated</td>
<td>- Add normal serum, BSA, non-fat dry milk, or detergent to buffer used as the primary antibody diluent. Be sure that the antibody diluent has sufficient salt to minimize nonspecific ionic interactions. Generally diluents should contain from 0.15M (0.9%) to 0.6M sodium chloride.</td>
</tr>
<tr>
<td><img src="image" alt="A" /></td>
<td>An Avidin/Biotin blocking (SP-2001) step will block off-target binding of ABC regardless of the cause.</td>
<td>• Add 2% or more normal serum from tissue species to the secondary antibody diluent, and/or reduce concentration of the secondary antibody.</td>
<td>- Primary Antibody</td>
<td>- Change source or species of primary.</td>
</tr>
<tr>
<td><img src="image" alt="A" /></td>
<td>Or, the following techniques can be used based on the specific cause.</td>
<td>• Use additional blocking agents such as 2% immunohistochemical grade BSA (SP-5050), nonfat dry milk, gelatin, or 0.1% detergent.</td>
<td>- Secondary Antibody</td>
<td>Some commercial diluents for the primary antibody can contribute to the background. Use diluent compatible with the detection system.</td>
</tr>
<tr>
<td><img src="image" alt="A" /></td>
<td>• Endogenous protein-bound biotin-Avidin/Biotin Blocking Kit (SP-2001)</td>
<td>Blocking serum from the wrong species was used.</td>
<td>- Biotin</td>
<td>If the section shows small, amorphous, punctate staining, the primary antibody may have some denatured precipitated immunoglobulin.</td>
</tr>
<tr>
<td><img src="image" alt="A" /></td>
<td>• Endogenous lectins – add 0.2M alpha-methyl mannoside (S-9005) to the ABC diluent</td>
<td>Use serum from the same species in which the secondary antibody was produced.</td>
<td>- Biotin</td>
<td>Centrifuge primary antibody; use supernatant.</td>
</tr>
<tr>
<td><img src="image" alt="A" /></td>
<td>• Ionic interactions – make up ABC Reagent in buffer containing 0.5M NaCl</td>
<td></td>
<td>- Biotin</td>
<td>Tissue sections dried out during procedure. Be sure to keep tissue sections moist during all steps in the procedure.</td>
</tr>
<tr>
<td><img src="image" alt="A" /></td>
<td>Certain grades of BSA may contain contaminants that can contribute to background staining.</td>
<td></td>
<td>- Biotin</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="A" /></td>
<td>If BSA is added to the blocking serum, use immunohistochemical grade BSA (SP-5050) or omit BSA. Avoid Fraction V-grade BSA.</td>
<td></td>
<td>- Biotin</td>
<td></td>
</tr>
</tbody>
</table>

Repeat Control A

Repeat Control B

Repeat Control C

Repeat Control D

Repeat Control D
If staining is weak or absent, use these tests.

### A - Enzyme/Substrate

**For Peroxidase Substrate:**
Add 1-2 drops of ABC Reagent to 1 ml peroxidase substrate working solution. Color of solution should change within about 5 seconds.

**For Any Substrate:**
Place 1 drop ABC Reagent on a small piece of nitrocellulose and then immediately dip the nitrocellulose into substrate. A colored spot will develop where the ABC Reagent was dotted.

If color develops, SEE B. If no color develops, SEE BELOW.

Deionized water can contain inhibitors of the peroxidase reaction. Even if the water has very low conductivity, the peroxidase reaction can be severely compromised.

Use glass distilled water for the preparation of the substrate solution.

Check the pH of the substrate buffer. Buffers of different pH values are recommended for different substrates. Use clean glassware to prepare substrate; traces of chlorine, cleaning solutions, etc. may inhibit the peroxidase reaction.

The substrate should be made according to instructions.

### B - Primary Antibody

Use the primary antibody at the optimal concentration. If activity of the primary is lost over time, a higher concentration may be required to achieve optimal staining. Treatments such as freeze/thawing, especially with monoclonal antibodies, may result in partial or complete inactivation of the antibody. High concentrations of antibodies may also reduce staining.

Testing the antibody on a known positive sample may provide information on the activity of the antibody. If the known positive sample is positive, but the test section is negative, SEE NOTES.

If the pH of the diluent for the primary antibodies is incorrect, the antibody may not bind well to the antigen.

Check the pH of the diluent. Generally TBS or PBS, pH 7.0-8.2, is recommended.

If the primary antibody recognizes an antigen in the diluent, it may bind to the antigen in solution rather than on the tissue section. Common diluent additives such as normal serum, fetal bovine serum, or nonfat dry milk may contain significant antigen concentrations that are recognized by the primary antibody.

Take care that the diluent for the antibody does not contain the antigen.

If negative, SEE C.

### C - Biotinylated Secondary Antibody

Inappropriately high dilutions of biotinylated secondary antibody can result in diminished staining.

Generally a 1:200 to 1:500 dilution of our biotinylated secondary antibodies will give optimal staining.

If the diluent contains any neutralizing antibodies, diminished staining could result. For example, biotinylated anti-mouse IgG should not be diluted in mouse serum. The immunoglobulins in mouse serum will bind the biotinylated anti-mouse and prevent this secondary antibody from binding to the primary antibody.

Remove source of neutralizing antibodies.

If the biotinylated antibody is incorrect, no staining will occur. The biotinylated antibody should be specific for the species in which the primary antibody is made. For example, biotinylated anti-rabbit IgG should be used with primary antibodies made in rabbit.

If negative, SEE NOTES.

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**NOTES:**

**Procedure Check**
An equal volume of Reagent A and then Reagent B should be added to a defined volume of buffer. Do not mix Reagent A and Reagent B and then dilute. This procedure may result in an inactive complex.

Avoid adding potential sources of biotin to the diluent for ABC. Serum, nonfat dry milk and culture media are common sources of biotin. Some grades of BSA may also interfere with the avidin/biotin interaction. **Avoid using Fraction V-grade BSA. If BSA is added, use only an immunohistochemical grade (SP-5050).**

**Blocking**
Some animals from which blocking serum was obtained may have developed antibodies to the antigen in question. If present, the antibodies may bind to the antigen and prevent the primary antibody from binding. **Try other blocking proteins such as an immunohistochemical grade of BSA, gelatin, fetal bovine serum, nonfat dry milk, etc. or 1% detergent.**

**Fixation Check**
Be sure that the method employed for preparing the sample is appropriate to preserve the primary antibody target antigen. **Use a high temperature antigen unmasking technique with an appropriate Antigen Unmasking Solution (Citrate-based, H-3300; or Tris-based, H-3301).**

**Counterstain/Mounting**
Some enzyme reaction products are soluble in alcohol, xylenes or other solvents used for non-aqueous permanent mounting. Be certain that the enzyme reaction product is compatible with the counterstain and mounting medium. **A substrate/counterstain compatibility chart is available on our website: www.vectorlabs.com**