TROUBLESHOOTING GUIDE

Each reagent in our VECTASTAIN® ABC and VECTASTAIN® Elite® ABC Kits has been carefully produced to ensure consistent and optimal staining. Reagents in every kit have been rigorously tested in our laboratories using a variety of immunohistochemical applications and are guaranteed to retain their activities during prolonged storage. However, even with the best systems, problems can occasionally arise. The tissue sections may have an inordinately high background stain; color development may occur in inappropriate locations in the section; or the desired staining may be substantially diminished or absent. Not all of the causes for aberrant staining may be obvious, and trying to solve the problem may become a time consuming and frustrating task.

We have developed this troubleshooting guide in the hopes that most of the common sources of problems encountered in immunocytochemical staining using the peroxidase or alkaline phosphatase-based VECTASTAIN® ABC Kits can be identified and corrected.

This guide is divided into three major parts. If inappropriate staining occurs, use the portion below; if weak staining or an absence of staining is the problem, refer to the back. The Notes section provides some general suggestions for optimizing your immunohistochemical procedure. If your problem cannot be solved using this guide, our technical service staff can be reached by phone: (650) 697-3600, fax: (650) 697-0339, or email: vector@vectorlabs.com.

If background staining occurs, use these controls on tissue sections.

A

- Substrate Alone

Staining

No Staining See B.

Endogenous enzyme may be developing the substrate.

- When running this control, use the same time and conditions that would be appropriate for antibody staining. Color that develops with long exposure to substrate may not be seen within the development time used for specific staining.
- If staining is of a generalized nature, it may be due to endogenous enzyme smeared across the section when the section was prepared.

Block endogenous enzyme appropriately:

Peroxidase - use 0.3% H_2O_2 in methanol for 30 minutes or 3% H_2O_2 in water for 5 minutes.

Alkaline Phosphatase - add levamisole to alkaline phosphatase substrate unless endogenous enzyme is the intestinal isoform.

For alternative techniques to these common blocking methods, visit our website, www.vectorlabs.com.

Repeat Control A

B

- Blocking Serum
- ABC Reagent
- Substrate

Staining

No Staining See C.

ABC reagent may be binding to tissues for three main reasons:

- Endogenous protein-bound biotin
- Endogenous lectins
- Ionic interactions

An Avidin/Biotin blocking step after the normal serum blocking step will eliminate the binding of the ABC regardless of the cause. Or, the following techniques can be used based on the specific cause:

- Endogenous protein-bound biotin– **Avidin/Biotin block**
- Endogenous lectins – **add 0.2M alpha-methyl mannoside to the ABC diluent**
- Ionic interactions – **make up ABC Reagent in buffer containing 0.5M NaCl**

Some protocols suggest the use of Bovine Serum Albumin (BSA) in the blocking procedure. Certain grades of BSA may contain contaminants (bovine IgG, lipids, etc.) that can contribute to background staining.

Use an immunohistochemical grade of BSA or omit BSA.

Repeat Control B

C

- Blocking Serum
- Biotinylated Secondary Antibody
- ABC Reagent
- Substrate

Staining

No Staining See D.

Cross-reactivity may occur between the biotinylated secondary antibody and endogenous immunoglobulins or other tissue proteins.

- This can occur when the secondary antibody recognizes identical or closely related amino acid sequences of these proteins.

Add 2% or more normal serum from tissue species to the biotinylated secondary antibody diluent, and/or reduce concentration of the biotinylated secondary antibody.

Biotinylated antibody may bind nonspecifically to tissue components.

Use additional blocking agents such as 2% immunohistochemical grade BSA, nonfat dry milk, gelatin, or 0.1% detergent.

The wrong species of blocking serum was used.

Use serum from the same species in which the biotinylated antibody was produced.

Egg white or egg white products were used for coating slides, diluting buffers, or blocking tissues.

Traces of avidin may be present which bind to the tissue section. Biotinylated secondary antibodies can bind to bound avidin resulting in unwanted staining.

Avoid using egg white proteins.

Repeat Control C

D

- Blocking Serum
- Primary Antibody
- Biotinylated Secondary Antibody
- ABC Reagent
- Substrate

Inappropriate Staining

Too much primary antibody has been used.

- The concentration of primary antibody should be the amount which produces clean specific staining without background.

Reduce primary antibody concentration.

The primary antibody may cross-react with other tissue epitopes or bind nonspecifically.

Add normal serum, BSA, non-fat dry milk, gelatin or detergent to primary antibody diluent.

Change source or species of primary antibody.

The diluent for the primary antibody contains little or no sodium chloride.

Be sure that the diluent for the primary antibody has sufficient salt to block nonspecific binding. Generally diluents should contain from 0.15M (0.9%) to 0.6M sodium chloride.

If the section shows small, amorphous, punctate staining, the primary antibody may have some denatured precipitated immunoglobulin.

Centrifuge primary antibody; use supernatant.

Tissue sections dried out during procedure.

Be sure tissue sections are kept moist during all steps in the procedure.

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 DAB, Vector® VIP, Vector® NovaRED™, or Vector® SG substrate 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 freshly diluted H_2O_2 to prepare substrate solution. The final H_2O_2 concentration should be about 0.01%. 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

Be sure primary antibody is used at an appropriate concentration and that it is active. If potency is lost over time, a higher concentration of primary antibody may be required to achieve optimal staining. Harsh treatment such as freeze/thawing, especially with monoclonal antibodies, may result in a partial or complete inactivation of the antibody. High concentrations of antibodies may also reduce staining.

Testing the antibody on sections taken from another known positive block may provide information on the activity of the antibody. If the known positive block 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 which is present in biological fluids, it may bind to the antigen in solution rather than on the tissue section. Common diluent additives that may contain significant antigen concentrations are normal serum, fetal bovine serum, or nonfat dry milk.

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 1:100 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.

Use correct biotinylated antibody.

If negative, SEE NOTES.

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.**

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

If a specific tissue section does not stain, but other sections stain using the same detection system, the antigen may have been destroyed by fixation or embedding. If possible, be sure that the method employed for preparing the section is appropriate to preserve antigen and provide access to all the detection system reagents. **Use an antigen unmasking technique employing heat or protease digestion to recover antigen damaged by fixation.**

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.**

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