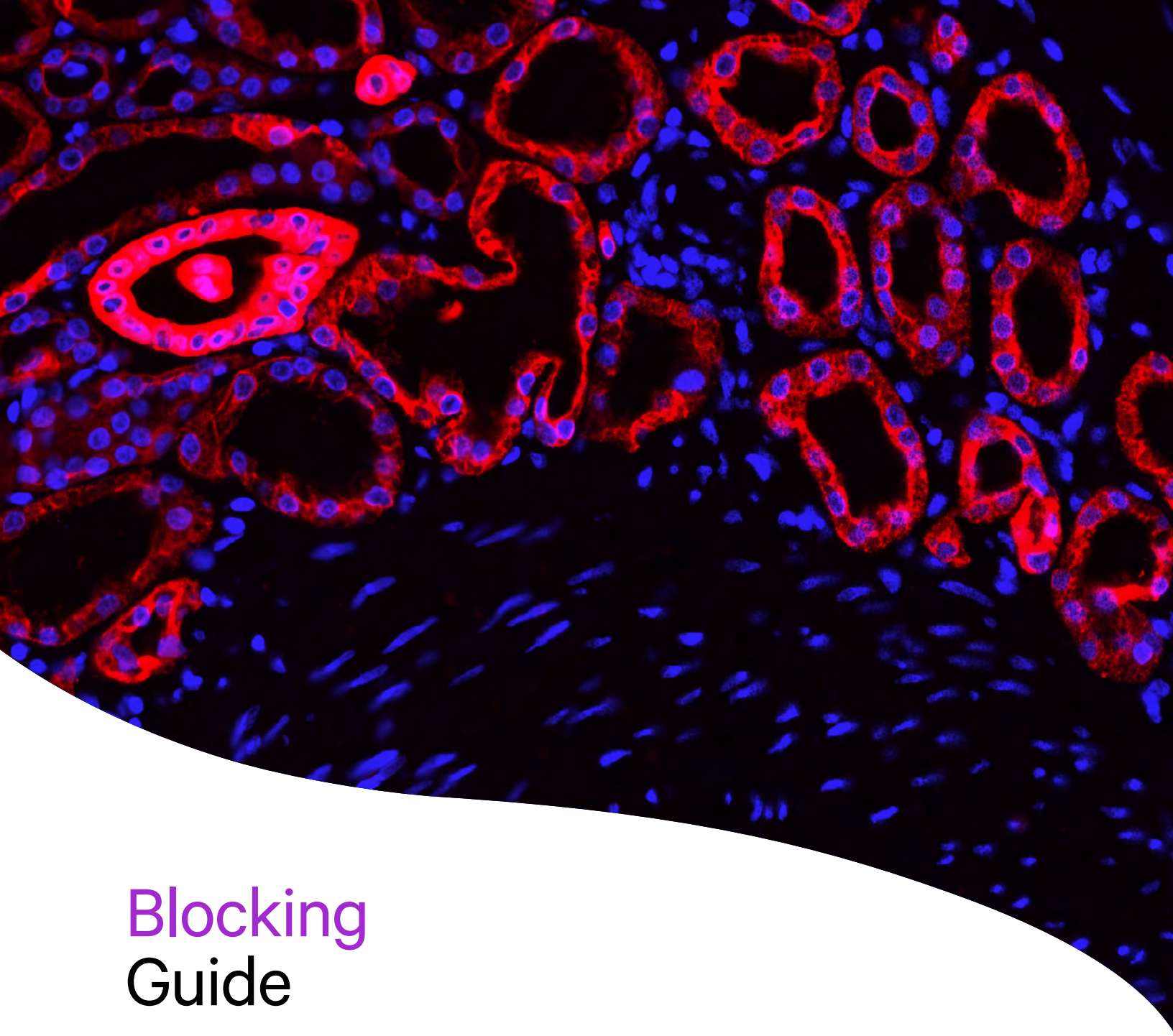

# Blocking Guide

Tips & tricks to improve your immunohistochemistry and immunofluorescence staining

vector LABORATORIES

# Table of contents



*Cover Image: Human kidney (FFPE) stained for AE1/AE3, red autofluorescence reduced using TrueVIEW® Autofluorescence Quenching Kit with DAPI (SP-8500).*

# Introduction

Performing a successful immunohistochemistry (IHC) or immunofluorescence (IF) experiment requires the perfect combination of primary antibody, secondary antibody, and detection system. Researchers need to puzzle through many different options for each component of this triad to find the combination that yields a clear, low-background, high-contrast staining. Equally important is keeping the wrong molecules out of the way. When non-specific interactions occur, high background or false-positive results often affect the quality of the final results.

Blocking is a key step to ensure low background and a clear staining of the target epitope. Many factors can contribute to high background and non-specific staining, including endogenous enzyme activity, endogenous biotin binding, endogenous immunoglobulin binding, cross-reactivity, and autofluorescence (Figure 1). When performed correctly, the blocking step reduces non-specific binding and quenches autofluorescence and endogenous enzyme activity, resulting in clear visualization of the target epitopes (1,2). Specific blocking methods and reagents vary according to the application, which reinforces the need to thoroughly consider all steps of the experiment to identify the most suitable approach. This guide provides practical solutions to each potential source of non-specific staining in IHC and IF applications.

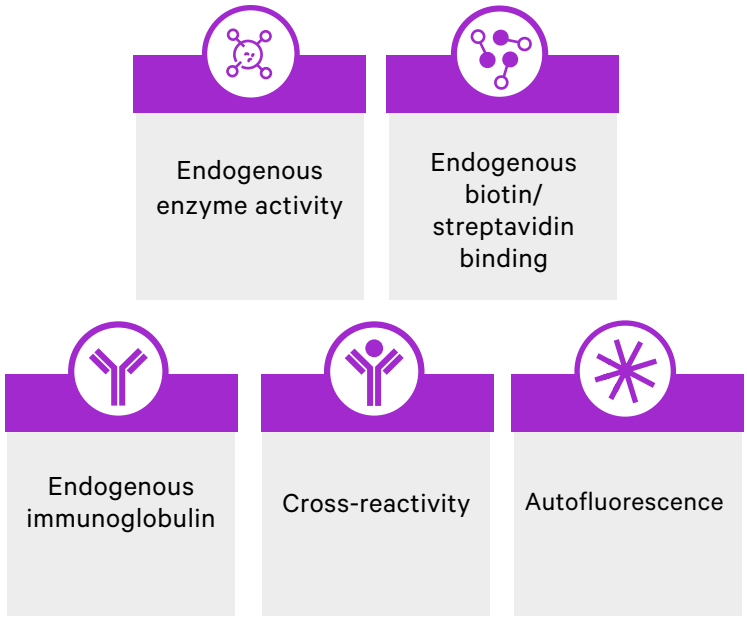


***Figure 1*** *Main factors contributing to high background and non-specific staining in IHC and IF experiments.*

# Endogenous enzyme activity

Many detection kits use secondary antibodies conjugated to reporter enzymes, such as horseradish peroxidase (HRP) and alkaline phosphatase (AP), that convert chromogenic substrates into colored precipitates. Alternatively, reporter enzymes can also be conjugated to the primary antibody. HRP, like any other peroxidase, catalyzes the transfer of electrons from a substrate to peroxide species, whereas AP promotes the hydrolysis of phosphate groups. In IHC applications, many different substrates can be used in combination with both enzymes to generate different colors. For example, the oxidation of diaminobenzidine (DAB) by HRP results in a brown precipitate, and the metabolism of nitroblue tetrazolium (NBT) by AP yields blue staining. A total of 9 different color tones are available for single and multicolor immunostaining.

Some tissues have high endogenous peroxidase (e.g., blood-heavy tissues such as the spleen and kidney) and phosphatase (e.g., kidney, intestine, and liver) (1). These endogenous enzymes can metabolize chromogenic substrates, resulting in high background in IHC applications, but adding specific reagents to the blocking steps can help mitigate the problem (Figure 2).

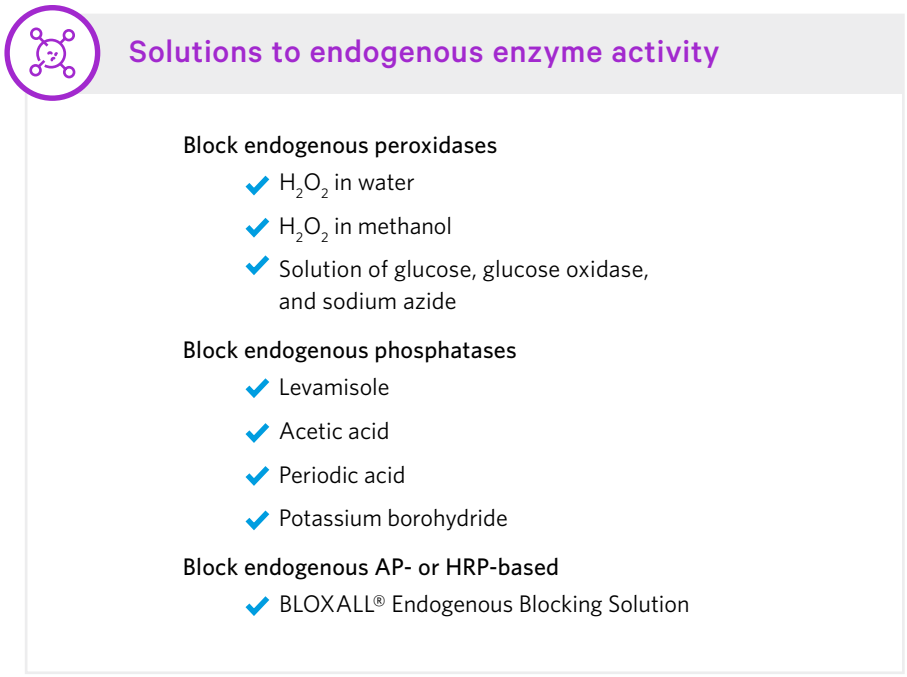


***Figure 2*** *Solutions to endogenous peroxidase and phosphatase activity.*

## Pro Tip

Hydrogen peroxide is a highly reactive compound that decomposes to oxygen and water. Storing $H_2O_2$ in the refrigerator can slow down its decomposition. Over time, regularly used $H_2O_2$ can lose its ability to inhibit peroxidase (even before expiration), and a fresh bottle can solve the problem. Researchers should exercise caution not to contaminate the stock $H_2O_2$, which can lead to accelerated decomposition and increase pressure in the container.

Using saturating amounts of hydrogen peroxide ($H_2O_2$) contributes to irreversible inhibition of endogenous peroxidase in the tissue specimen. A solution of 3% $H_2O_2$ in water is a good general block and the most rapid and straightforward technique for quenching endogenous peroxidase activity. Incubating the sections with this solution for 5 minutes followed by a 2- to 3-minute wash in water is sufficient, in most cases, to inactivate tissue enzymes completely. However, this approach can generate intense bubbling, contributing to morphological damage of frozen sections and specimens with large amounts of endogenous enzyme activity (e.g.s and blood smears or sites with inflammatory infiltrate). Alternatively, incubating the specimen with a solution of 0.3% $H_2O_2$ in methanol for 20 to 30 minutes also results in effective peroxidase inactivation. Methanol cleaves heme groups present in peroxidase enzymes, and, for this reason, a much lower concentration of $H_2O_2$ produces adequate results (3). If results are unsatisfactory, the investigator can double the concentration of $H_2O_2$ and/or shorten the incubation time as appropriate for the specimen. Blocking peroxidase with $H_2O_2$, even in lower concentrations, can damage the epitope and reduce the antigenicity of cell surface markers such as CD4 and CD8 (4). When staining these markers, blocking should be performed after incubation with primary antibody.

Another solution without $H_2O_2$ in its formulation (180 g β-D-glucose, 5 mg glucose oxidase, and 6.5 mg sodium azide in 50 ml PBS) also results in effective quenching of peroxidase activity. Incubating sections with this solution for 1 hour at 37 °C slowly and steadily produces very low concentrations of $H_2O_2$ by enzymatic reaction, leading to consistent and complete inhibition of peroxidase activity (5).

Endogenous AP can also metabolize IHC substrates and contribute to non-specific staining. The most common solution to this problem includes incubating sections with levamisole 10 mM (6,7). This reagent reversibly inhibits most forms of AP, except for the intestinal isoenzyme. Because IHC staining kits usually use intestinal AP as the reporter enzyme, levamisole can simply be added to the substrate mix to effectively inhibit endogenous AP activity. When an IHC experiment requires inactivation of the intestinal isoform, incubating the sections with 20% acetic acid for 15 minutes at 4 °C yields adequate results. However, this process needs to be performed before incubation with antibodies (7). A final option for blocking endogenous phosphatases includes treating the sections with 2.3% periodic acid for 5 minutes followed by 0.02% potassium borohydride for 2 minutes (8).

## Blocking for immunohistochemistry

Endogenous peroxidase present in tissue sections can result in background when using an HRP detection system if this activity is not quenched. In this video tutorial we demonstrate how to block endogenous peroxidase using a hydrogen peroxide incubation procedure.

Watch now ▶


As a simple, straightforward solution to quenching endogenous enzyme activity, Vector Laboratories offers BLOXALL Endogenous Blocking Solution, a reagent that inactivates endogenous peroxidase, pseudoperoxidase, and alkaline phosphatase in a single 10-minute incubation step. BLOXALL Endogenous Blocking Solution is ready to use and compatible with formalin-fixed, paraffin-embedded (FFPE) tissue sections, frozen tissue sections, and cell preparations.

# Endogenous biotin/ streptavidin binding

## Did you know?

Biotin, also known as vitamin B7, is a cofactor for some reactions that mostly occur in the mitochondria and is essential for the metabolism of fatty acids, glucose, and amino acids. Tissue types with higher mitochondrial activity, such as kidney, liver, spleen, and certain tumors, have high levels of endogenous biotin.

The strong affinity between avidin/streptavidin and biotin constitutes the basis of Avidin-Biotin Complex (ABC)-based detection systems. Biotin is a cofactor essential for the metabolism of carbohydrates, fats, and proteins and, thus, is present in many but not all cells. Avidin is a glycoprotein with a natural affinity for biotin, and the strong non-covalent bond between them has allowed the development of many assays, including ABC staining kits. In IHC detection systems, biotin is often conjugated to both the secondary antibody and the reporter enzyme (HRP or AP) of choice. Avidin, which has multiple binding sites, is then used to bind the biotinylated reporter enzyme to the biotinylated secondary antibody, forming a strong complex that resists changes in pH and temperature (9).

Streptavidin is another naturally occurring protein isolated from *Streptomyces avidinii* that also binds to biotin and is used in detection systems. The primary difference between avidin and streptavidin is that the latter is not glycosylated and does not bind to endogenous lectins. However, both avidin and streptavidin can bind to other macromolecules inside the cell through direct affinity (e.g., endogenous biotin) or non-specific binding due to charge/ionic interactions. Specific blocking reagents can prevent the binding of avidin/streptavidin to molecules that are not biotinylated, ensuring a clear and specific staining (Figure 3).

### Solutions to endogenous biotin binding

**Block endogenous biotin, biotin receptors, and avidin binding site**

- ✔ Biotin/Avidin blocking kit

**Block non-specific binding of avidin due to ionic interactions**

- ✔ Prepare reagents in buffer containing 0.5 M NaCl

***Figure 3*** *Solutions to endogenous biotin binding activity.*

Avidin/streptavidin and biotin blocking kits block all endogenous biotin, biotin receptors, and avidin/streptavidin binding sites in the specimen. In this way, avidin/streptavidin and biotinylated reporter enzymes from the IHC detection system will only bind to the biotinylated secondary antibody, and non-specific binding will not occur. In addition, dilution of ABC reagents in a buffer containing NaCl 0.5 M prevents non-specific binding of avidin/streptavidin due to charge/ionic interactions with the specimen.

# Endogenous immunoglobulin

### Pro Tip

Sometimes, M.O.M. and H.O.H. experiments do not yield high background; thus, it is important to always run pilot tests before trying complex solutions. Certain steps in the sample preparation process, such as perfusion with PBS before fixation, may significantly reduce endogenous immunoglobulin in the tissue.

If any of the detection antibodies in an IHC or IF procedure is directed against the species of a chosen specimen, undesired binding to endogenous immunoglobulin can occur and obscure the desired staining. Common examples of this scenario include the use of mouse primary antibody on mouse tissue (mouse on mouse, M.O.M.®, staining) and human or humanized primary antibodies on human tissue sections (human on human, H.O.H.™, staining).

Achieving a consistent and straightforward solution to this problem is challenging. The recommended approach includes combining primary and secondary antibodies and using serum from the same species as that of the tissue to quench unbound secondary antibodies. The antibody complex is then applied to the specimen (Figure 4) (10). The challenge with this approach is that all three components—primary antibody, secondary antibody, and normal serum—should be added at the optimal concentrations and ratios to enhance signal-to-noise adequately. In addition, the exact ratio between reagents differs based on the primary antibodies (10).

Vector Laboratories offers M.O.M. and H.O.H. immunodetection kits, eliminating the need for complicated and time-consuming protocol optimization steps (Figure 4). Each reagent is provided at the ideal ratio and concentration to effectively form and adsorb the antibody complex and deliver consistent results. M.O.M. and H.O.H. kits produce clear, crisp, specific, and low-background staining of target antigens on frozen and paraffin-embedded tissue sections.

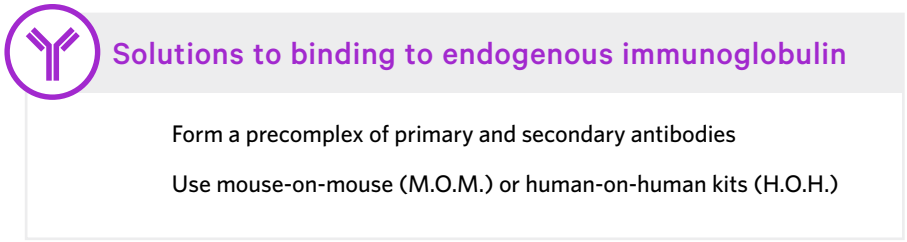


***Figure 4*** *Solutions to binding to endogenous immunoglobulin.*

# Cross-reactivity

## Pro Tip

Titration of primary and secondary antibodies is essential to achieving outstanding results with IHC and IF experiments. Here are a few tips on how to titrate an antibody:

- Select a positive control to be used across the whole titration process
- Check the product data sheet for recommended dilution
- Determine a series of dilutions that include the datasheet recommendation and more and less concentrated dilutions. For example, if the suggested dilution is 1:100, prepare the antibody at 1:25, 1:50, 1:100, 1:200, and 1:400.
- Analyze the data
- If necessary, test additional dilutions using the same positive control.

Cross-reactivity occurs when either the primary or the secondary antibody binds to an unintended epitope. Primary antibodies frequently bind to non-target sites in the specimen during the incubation process. These non-specific interactions rarely affect the final IHC results because they are weak and usually break during subsequent washes. However, when the primary antibody concentration is too high, non-specific binding of primary antibodies to non-target sites can persist, resulting in high background (2,4). An easy solution to this problem is reducing the primary antibody concentration (Figure 5). In fact, antibody concentration should be titrated to identify the concentration range that will yield optimal results—if it is too diluted, it can contribute to false-negative results, but, if it is too concentrated, it can promote non-specific binding.

Additional approaches can help reduce the binding of primary antibodies to non-target sites as titration alone might not solve the problem (Figure 5). Adding or increasing the concentration of blocking reagents to the buffer used as the primary antibody diluent may provide an efficient solution. Common choices include normal serum, bovine serum albumin (BSA), non-fat dry milk, or detergent. In addition, the antibody diluent should contain sufficient salt—from 0.15 M (0.9%) to 0.6 M (3.6%) sodium chloride—to minimize non-specific ionic interactions.

### Solutions to cross-reactivity

Reduce primary antibody concentration

Use a different primary antibody

Reduce secondary antibody concentration

Use secondary antibody adsorbed for the tissue species

Add or increase the concentration of

- ✔ BSA
- ✔ Non-fat dry milk
- ✔ Gelatin
- ✔ Detergent 0.1%
- ✔ Normal serum from secondary antibody host species
- ✔ Sufficient salt—0.15 M to 0.6 M

***Figure 5*** *Solutions to cross-reactivity.*

## Pro Tip

Different protein blockers for IHC and IF are available, and in general, they all help reduce the non-specific binding of antibodies. So what blocker should a researcher use in an experiment? Here are a few tips to help the empirical selection of blockers.

- Normal serum helps prevent non-specific binding of the secondary antibody to unintended targets. It should come from the same species of the secondary antibody and is usually used at 1–5%.
- Albumin is already present in the normal serum and reduces non-specific binding by competing with the antibody for the unintended target. When normal serum alone is insufficient to reduce background, adding extra BSA at 1–5% can help mitigate the problem.
- Gelatin and non-fat dry milk act similarly to BSA and can provide additional blocking of unintended targets. They are also used at 1–5%. However, non-fat dry milk cannot be used with antibodies against phosphoproteins and ABC-based detection systems.
- The best combination and concentration of blockers should be empirically determined for each application. Include a negative control to assess background.
- Blockers should be immunohistochemical grade and free from precipitates.

Alternatively, changing the primary antibody might significantly improve the results (1,4). Lower cross-reactivity can be achieved with a new combination of a primary antibody that detects the same target but is from a different host species and a compatible secondary antibody. In addition, using a monoclonal instead of a polyclonal primary antibody increases specificity and lowers background. One final approach is using a different monoclonal antibody that binds to another epitope on the same target protein.

Although primary antibodies can bind to non-target sites, cross-reactivity is more commonly observed between secondary antibodies and unintended epitopes on the sample. Immunoglobulins from different species can share homologous sequences and have similar quaternary structures. For example, anti-mouse secondary antibodies can bind to rat endogenous immunoglobulins present in the tissue. However, this type of cross-reactivity is not limited to closely related species and is not a variable that can be easily predicted. Using secondary antibodies that have been adsorbed for the chosen tissue species effectively reduces cross-reactivity (Figure 5). Cross-adsorption is a purification step in which a solid phase is used to filter the antiserum and remove antibodies reacting to immunoglobulins from unintended species. This additional process effectively increases specificity and decreases cross-reactivity in secondary antibodies. The product data sheet usually informs the user if the antibody has been adsorbed for a specific species.

Other approaches can help reduce cross-reactivity when an adsorbed secondary is unavailable for a particular application (Figure 5). In general, the solutions to mitigate primary antibody cross-reactivity also apply to secondary antibodies. Titrating the secondary antibody and potentially reducing its concentration is an all-around good practice. Blocking reagents, such as normal serum, BSA, non-fat dry milk, gelatin, or 0.1% detergent, can also reduce the binding of secondary antibodies to non-target sites. Because choosing the wrong blocking serum can worsen the problem of cross-reactivity, normal serum needs to originate from the same host species as the secondary antibody. Caution is also important when using BSA. This blocking reagent can cross-react with anti-goat antibodies and should be immunohistochemical grade.

Additional antibody-related problems can contribute to non-specific staining in IHC and IF. If the section displays small, amorphous, punctate staining, the primary antibody might contain some denatured precipitated immunoglobulin. Centrifuging the primary antibody and using the supernatant usually solves this problem.

## Getting to the specifics: Blocking for immunohistochemistry

When performing IHC, scientists aspire to reduce background or non-specific staining on the specimen. Explore how blocking the sources of non-specific signals increases the signal-to-noise ratio for optimal results.

Read more ▶

# Autofluorescence

Autofluorescence, another potential cause of background in IF, occurs when molecules other than the antigen-bound antibody complex emit fluorescence. Common natural sources of autofluorescence include the heme groups of red blood cells, collagen, elastin, NADH, and lipofuscin. Autofluorescence from these sources makes the interpretation of IF results challenging, particularly when using the green and red channels (11–13). The fixation process can also contribute to autofluorescence as formalin-induced cross-links can emit fluorescence in a broad range of wavelengths (11).

The common solution to autofluorescence is the use of dyes, such as Sudan black, trypan blue, pontamine sky blue, crystal violet, and DAB, to quench unwanted fluorescence through absorption of incident radiation (Figure 6) (14–16). However, not all of these dyes will reduce fluorescence from every source. For example, Sudan black reduces lipofuscin-related autofluorescence but is ineffective in quenching signal from formalin-induced cross-links, collagen, and red blood cells. A combination of different dyes might help to quench autofluorescence from lipofuscin and other sources completely (17). In addition to using fluorescence-quenching dyes, adding 1–10 mM copper sulfate in 50 mM ammonium acetate buffer to the final wash helps to reduce lipofuscin-related autofluorescence (18). One final note of caution is that these dyes will reduce both non-specific and targeted fluorescence (17).

## Solutions to autofluorescence

**Use hydrophobic dyes**

- ✔ Crystal violet
- ✔ DAB
- ✔ Sudan black
- ✔ Pontamine sky blue
- ✔ Trypan blue

**Solution of copper sulfate with ammonium**

**Add Tween-20 washes**

**Switch from FFPE to frozen tissue**

**Switch from fluorescent secondary to AP- or HRP-based detection system**

**Use TrueVIEW® Autofluorescence Quenching Kits to quench autofluorescence**

***Figure 6*** *Solutions to autofluorescence.*

In addition to using quenching dyes, modifying some steps in the IF workflow can help minimize autofluorescence: Using a different fixative when working with blood-rich specimens; switching from formalin-fixed, paraffin-embedded preservation of tissues to cryopreservation; adding Tween-20 to the wash buffer. Dropping fluorescence altogether and using HRP- or AP-based detection systems is also a practical solution when all other alternatives fail.

Vector Laboratories also offers TrueVIEW Autofluorescence Quenching Kits, an innovative solution to eliminating autofluorescence. Unlike the dyes previously mentioned, these quenching kits contain a hydrophilic molecule that effectively reduces autofluorescence from non-lipofuscin sources: aldehyde fixation, red blood cells, collagen, and elastin. In addition, TrueVIEW Autofluorescence Quenching Kits selectively reduce unwanted fluorescence and enhance signal-to-noise, resulting in clear images with a "true view" of the target antigen.

## Reduce tissue autofluorescence and dramatically enhance signal-to-noise ratio

Autofluorescence can make it difficult or impossible to distinguish antigen-specific signal from non-specific background. Learn how you can reduce autofluorescence in your immunofluorescence workflow.

Read more ▶

## Learn how to access the clear, unambiguous view of antigen expression you need for accurate data interpretation.

### Optimize your immunofluorescent staining—Tips to overcome background interference

Interfering background signal in immunofluorescence can hamper your ability to accurately distinguish real target antigen expression. Situations where the expected expression pattern is unknown—whether due to transient protein expression, modified expression patterns due to disease states or a treatment model, or unknown levels of target presence due to species and tissue type—increase analysis complexity. Learn how to access the clear, unambiguous view of antigen expression you need for accurate data interpretation.

Explore tips to improve your immunofluorescent staining to help you achieve consistent, reliable data.

Watch now ▶

# Putting it all together

Running deletion controls is the optimal approach to identify the source of background and the ideal blocking solution. Figure 7 provides an overview of the steps for performing deletion controls and highlights potential challenges and solutions in different scenarios.

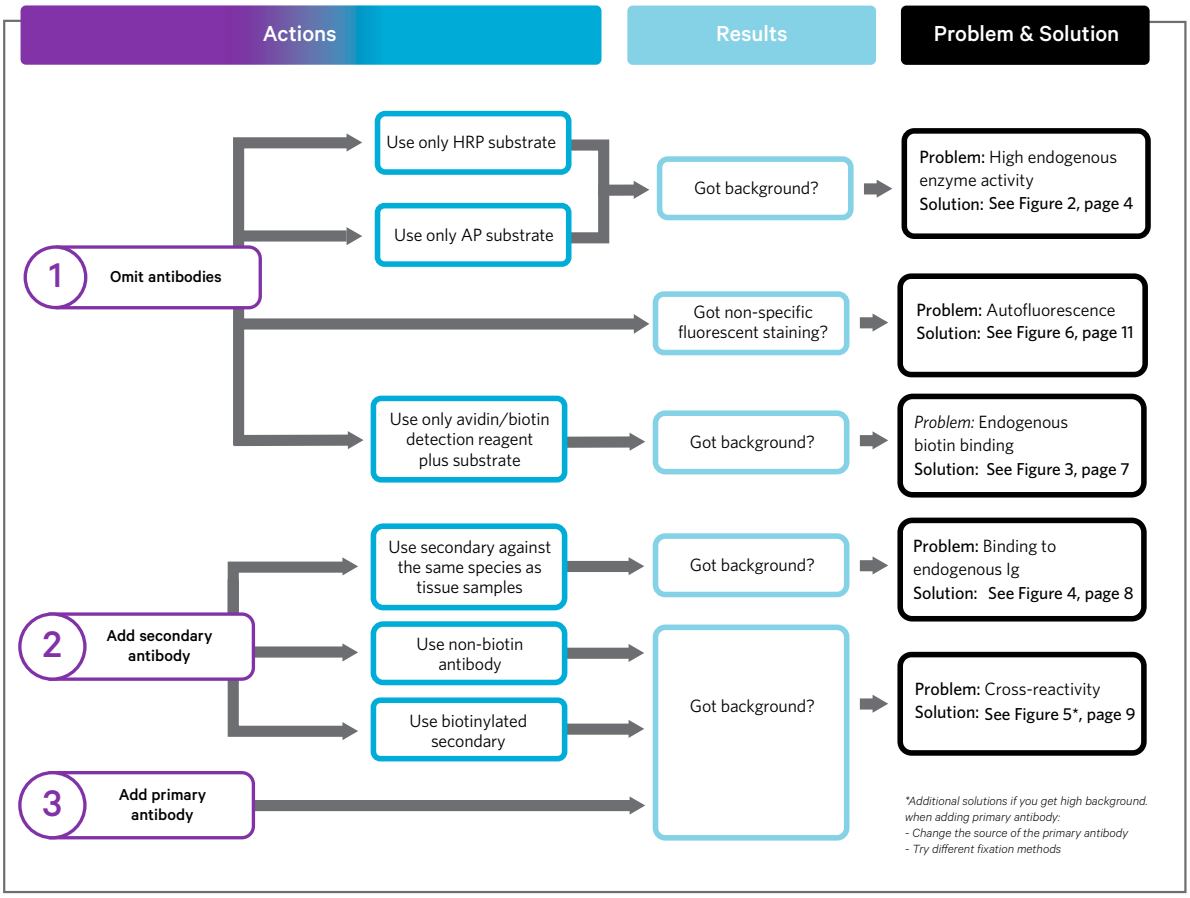


***Figure 7*** *Diagram of the steps in the deletion control process and possible challenges and solutions for each scenario.*

# Product selection guide

| Product | Cat. No. |
|---|---|
| BLOXALL Endogenous Blocking Solution, Peroxidase and Alkaline Phosphatase | SP-6000-100 |
| Levamisole Solution | SP-5000-18 |
| Avidin/Biotin Blocking Kit | SP-2001 |
| Streptavidin/Biotin Blocking Kit | SP-2002 |
| Anti-Streptavidin Antibody, DyLight™ 488 | SP-4488-.5 |
| Anti-Streptavidin Antibody, Biotinylated | BA-0500-.5 |
| Anti-Streptavidin Antibody, Unconjugated | SP-4000-1 |
| M.O.M. (Mouse on Mouse) Elite® Immunodetection Kit, Peroxidase | PK-2200 |
| M.O.M. (Mouse on Mouse) ImmPRESS® HRP (Peroxidase) Polymer Kit | MP-2400 |
| M.O.M. (Mouse on Mouse) Immunodetection Kit, Basic | BMK-2202 |
| M.O.M. (Mouse on Mouse) Immunodetection Kit, Fluorescein | FMK-2201 |
| H.O.H. (Human on Human) Immunodetection Kit | HOH-3000 |
| Bovine Serum Albumin (BSA), Immunohistochemical Grade | SP-5050-500 |
| Vector® TrueVIEW® Autofluorescence Quenching Kit | SP-8400-15 |
| Vector® TrueVIEW® Autofluorescence Quenching Kit with DAPI | SP-8500-15 |

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