

FAQ – Why do I have high background or non-specific staining in IHC/IHC-P?

Primary / secondary antibody concentration may be too high

The primary and secondary antibody concentrations recommended in SYSY Antibodies reference protocols yield reliable staining under our validated conditions. However, IHC and IHC-P results are highly sensitive to multiple variables - including fixation and antigen retrieval methods, primary and secondary incubation times, blocking reagents, detection systems, and tissue type. Consequently, the optimal antibody concentrations must be determined experimentally for each application. SYSY Antibodies therefore recommends titrating both primary and secondary antibodies for every tissue type to ensure robust and reproducible staining.

Endogenous enzyme interference when using chromogenic detection

Tissues such as kidney and liver contain high levels of endogenous peroxidases, which may lead to false-positive staining in HRP-based detection systems. To prevent this, block endogenous peroxidase activity using 3% H₂O₂. Note that some antigens are sensitive to high concentrations of hydrogen peroxide; in such cases, perform the H₂O₂ block after primary antibody incubation to preserve antigen integrity.

Endogenous alkaline phosphatase (AP) is present in tissues such as intestine, kidney, and lymphoid organs, and can be effectively inhibited with 1 mM levamisole when using AP-based detection methods.

SYSY Antibodies recommends assessing endogenous enzyme activity by running a control in which the primary and secondary antibodies - and the ABC complex, if applicable - are omitted from the protocol.

Endogenous biotin when using the ABC method

Certain tissues, such as kidney and liver, contain high levels of endogenous biotin. This can cause substantial background or false-positive staining when using the ABC method, particularly after antigen retrieval with Tris-EDTA or other EDTA-based buffers. SYSY Antibodies therefore recommends including an avidin-biotin blocking step when employing the ABC detection system. If background staining persists, consider switching to a polymer-based detection system as an alternative to the ABC method.

To assess the contribution of endogenous biotin, SYSY Antibodies recommends running a control in which only the avidin-biotin complex is applied, while the primary antibody and the biotinylated secondary antibody are omitted.

Endogenous biotin when using fluorescent detection

Fluorophore-conjugated avidin or streptavidin can bind to endogenous biotin, leading to nonspecific background staining. For tissues with high endogenous biotin levels, SYSY Antibodies therefore recommends including an avidin-biotin blocking step when using these reagents.

To evaluate endogenous biotin interference, SYSY Antibodies recommends running a control in which only the fluorophore-conjugated secondary reagent is applied while the primary antibody is omitted.

Sections have dried out

Make sure that the sections do not dry out. Use a humidified chamber for the incubation of slide mounted sections.

When working with free-floating sections, verify that they are fully submerged in buffer throughout all incubation steps.

Secondary antibody causes background staining

Secondary antibodies can cross-react with endogenous immunoglobulins in the tissue or generate false-positive signals through nonspecific binding. Monoclonal antibodies against mouse proteins are often generated in rat to avoid classic “mouse-on-mouse” issues. However, when using anti-rat secondary antibodies on mouse tissue, it is essential that these secondaries are mouse-adsorbed to minimize background staining. Efficient perfusion before fixation is essential. Residual blood contains high amounts of endogenous antibodies that can be bound by secondary antibodies resulting in unspecific blood vessel staining.

In multiplex experiments, species-specific secondary antibodies may also cross-react with primary antibodies raised in other species. To minimize these issues, use secondary antibodies that are pre-adsorbed not only against immunoglobulins of the target species but also against IgGs from the host species of all other primary antibodies used in the experiment.

SYSY Antibodies therefore recommends evaluating potential cross-reactivity by omitting the primary antibody in a control staining. Additionally, perform lot-to-lot comparisons for all secondary reagents to ensure consistent performance.

Blocking is insufficient

For vibratome and cryostat sections, SYSY Antibodies advises using normal serum, as it offers a more reliable blocking effect than BSA. Ensure that the serum is derived from the host species of the secondary antibody to reduce background staining.

For formalin fixed paraffin embedded (FFPE) tissues SYSY Antibodies recommends using Agilent's serum-free protein block and antibody diluent (which also provides additional blocking) in its IHC-P reference protocols for most reliable performance.

When using alternative blocking reagents, carefully optimize blocking conditions to achieve performance comparable to the recommended reagents.

Autofluorescence

Autofluorescence occurs independently of specific immunofluorescent signals and can mask target detection, thereby compromising data interpretation. The main contributors are endogenous tissue components (e.g., erythrocytes, collagen, elastin, lipofuscin) and chemical modifications introduced by aldehyde fixation and paraffin embedding. Because autofluorescent molecules have their own excitation and emission spectra, unstained controls are useful for identifying them. Most autofluorescent compounds exhibit broader excitation and emission spectra than the fluorescent probes used for staining. Formaldehyde fixation, for example, induces autofluorescence across the 350–650 nm range, with an emission maximum at 500–550 nm. Lipofuscin also covers a broad spectrum, but its emission maximum lies around 600 nm. Several strategies can reduce autofluorescent signals, including chemical quenching (e.g., Sudan Black B), photobleaching, spectral unmixing, and fluorescence-lifetime imaging microscopy (FLIM).

Tissue sections too thick (IHC-P/FFPE)

Cut tissue sections thinner. FFPE tissue sections should be 2.5 - 5 μ M.