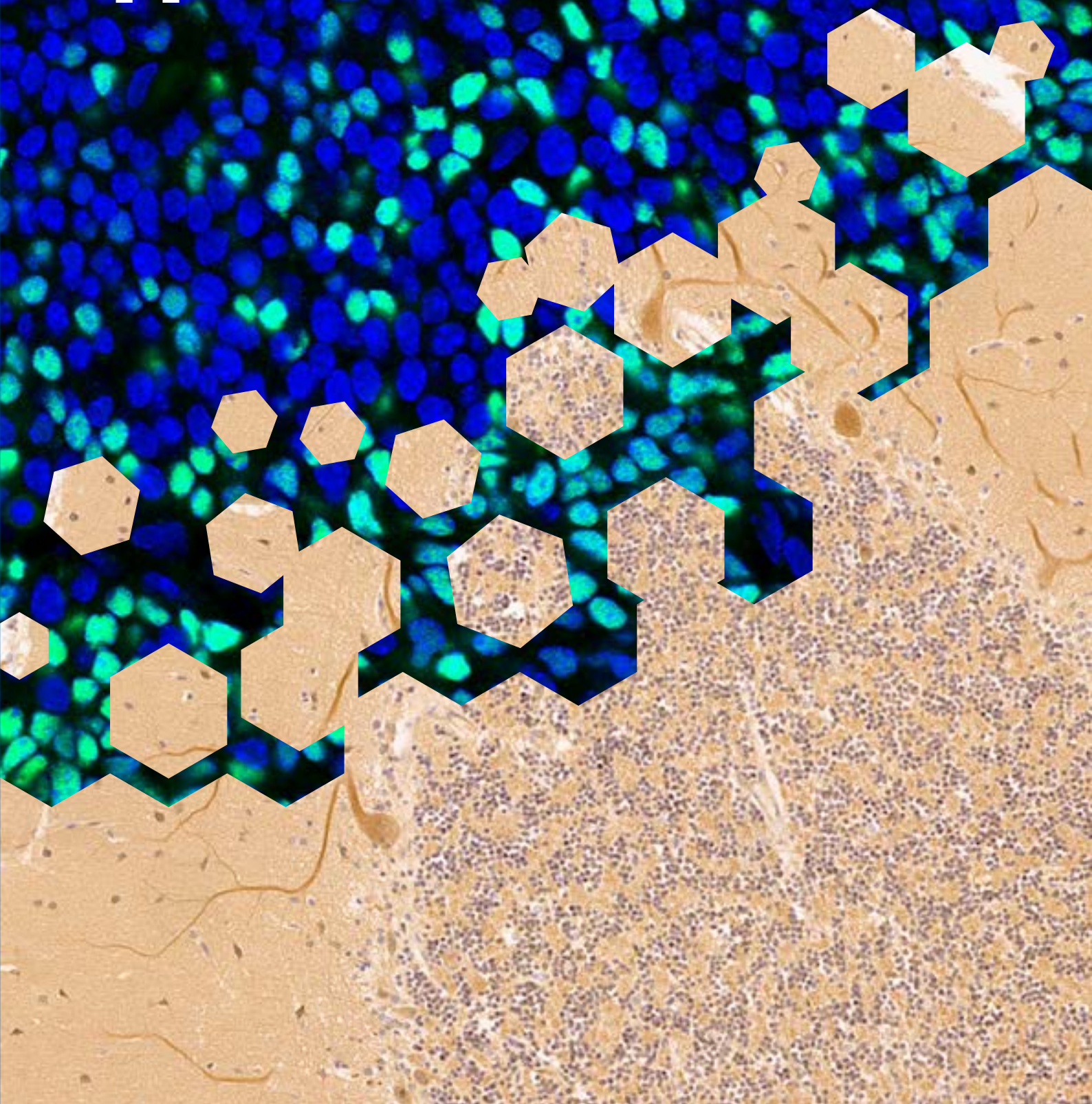


abcam

Immunohistochemistry Application Guide



Contents

Introduction	4
Tissue processing, fixation, and sectioning	5
Antigen retrieval	7
Blocking proteins	9
Blocking endogenous enzymes	10
- Peroxidase blocking	10
- Alkaline phosphatase blocking	10
The primary antibody	11
- Direct vs indirect detection	11
- Choosing a primary antibody	11
- Antibody specificity	11
- Prior use in IHC	12
- Clonality	12
Detection and amplification systems	13
- Chromogenic vs fluorescent detection	14
- Enzymes and chromogens	15
Multicolor IHC	16
Counterstaining	17
Controls for IHC	19
- Antigen (tissue) controls	19
- Reagent controls	19
Troubleshooting IHC experiments	20
- No staining	20
- High background	21
- Non-specific staining	22
- Poorly resolved or damaged tissue morphology	22

Introduction

Immunohistochemistry (IHC) uses antibodies to detect the location of proteins and other antigens in tissue sections. The antibody-antigen interaction is visualized using either chromogenic detection with a colored enzyme substrate, or fluorescent detection with a fluorescent dye.

Although less quantitative than assays such as western blotting or ELISA, IHC gives invaluable information about protein localization in the context of intact tissue. Protein expression patterns are tremendously valuable for pathologists and as diagnostic tools.

Essential to a successful IHC experiment is a robust, optimized, and reproducible staining regimen that makes use of high-quality, specific reagents.

IHC Resources

www.abcam.com/IHC-kits-and-reagents
www.abcam.com/IHC-primary-antibodies
www.abcam.com/IHC-secondary-antibodies

Tissue processing, fixation, and sectioning

Tissue fixation preserves antigens and prevents the autolysis and necrosis of harvested tissues. Embedding tissue provides support during sectioning and makes sections more robust.

The first decision when planning an IHC study is how to prepare the tissue sections. The most common method uses paraffin embedding. Frozen sections and floating sections are other options – each method has advantages and limitations (Table 1).

Sample fixation is key to tissue processing and is critical to prevent the degradation of antigens, cells, and tissue. Solutions of 10% buffered formalin and 4% formaldehyde (also called paraformaldehyde) are typical fixatives – these are near identical; formalin is a 40% solution of formaldehyde.

It is critical to fix or freeze samples quickly and thoroughly after harvesting and to ensure that samples are not too large to fix completely or freeze quickly.

Tissue processing resources

www.abcam.com/tissue-processing

Table 1. Paraffin vs freezing vs floating for IHC.

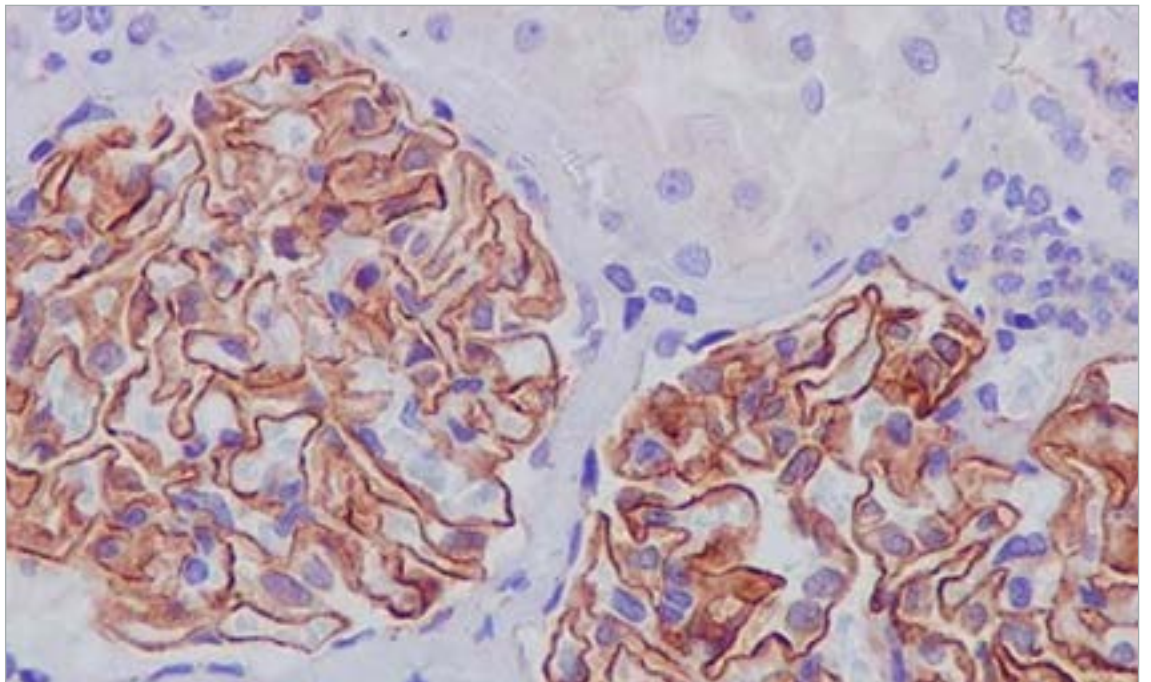
	Paraffin-embedded tissue	Frozen tissue	Floating sections
Fixation	Pre-embedding: formaldehyde.	Pre or post-sectioning: formaldehyde, methanol, ethanol, or acetone.	Pre-sectioning: formaldehyde.
Embedding / freezing	Tissue dehydrated and cleared before adding paraffin (pre-heated to 60°C) and left overnight.	Tissue frozen by immersion in liquid nitrogen, isopentane or by burying the sample in dry ice. Snap-freezing is common when detecting post-translation modifications such as phosphorylation.	Embedding not required.
Sectioning	Microtome	Cryostat	Vibratome
Storage	Multiple years at room temperature.	1 year at -80°C (longer at -190°C).	In cryoprotectant at -20°C, or short-term in PBS + azide at 4°C.
Advantages	Easy to handle without damaging the section.	Preserves enzyme function and antigenicity. Shorter protocol (lengthy fixation step usually not required).	Used with thicker sections (>25 µm): allows greater analysis of the 3D structure of the tissue.
Limitations	Over-fixation can mask the epitope – increased requirement for antigen retrieval. Lengthy processing: eg gradual dehydration in alcohol series and xylene to allow paraffin penetration.	Formation of ice crystals may negatively affect tissue structure if tissues are not frozen rapidly. Sections produced are often thicker than paraffin sections, increasing the potential for lower resolution and poorer images. May need to block active endogenous enzymes.	More challenging to image smaller structures and individual cells. Additional tissue clearing methods, such as CLARITY, may be required to reduce light scattering and image thicker sections.

Antigen retrieval

Perform antigen retrieval on formaldehyde-fixed tissue sections to expose antigenic sites and allow antibodies to bind.

Formaldehyde fixation results in protein cross-linking (methylene bridges), which masks epitopes and can restrict antigen-antibody binding. Antigen retrieval methods (Table 2) break these methylene bridges and expose antigenic sites, allowing antibodies to bind.

Frozen tissue sections are often not robust enough to be used with antigen retrieval without damaging the section. Many people tend to avoid using formaldehyde fixatives with frozen sections (or they are used with greatly reduced exposure time), thereby removing/reducing the need for antigen retrieval.



IHC for PDGFR beta in human kidney labeled anti-ZO1 tight junction protein (EPR19945-296) (ab221547). Heat mediated antigen retrieval was performed using Tris/EDTA buffer, pH 9.0 (ab93684).

Table 2. Primary methods of antigen retrieval.

	Heat-induced epitope retrieval	Proteolytic-induced epitope retrieval
Advantages	Gentler epitope retrieval and more definable parameters.	Useful for epitopes that are difficult to retrieve.
pH	pH 6 buffers are often used, but high pH buffers are widely applicable. Optimal pH must be determined experimentally.	Typically pH 7.4.
Temperature	Approximately 95°C.	Typically 37°C.
Incubation time	10–20 minutes.	10–15 minutes.
Buffer composition	Depends on pH required for the target antigen. Popular buffer solutions include sodium citrate, EDTA, and Tris-EDTA.	Neutral buffer solutions of enzymes such as pepsin, proteinase K or trypsin.
Precautions	Heating with microwaves can result in uneven epitope retrieval due to hot and cold spots. Rigorous boiling can lead to tissue dissociation from the slide.	Enzymatic retrieval can sometimes damage the morphology of the section – concentration and timing need to be optimized.

Antigen retrieval resorces

www.abcam.com/antigen-retrieval-buffers
www.abcam.com/antigen-retrieval-protocol

Blocking proteins

Block with sera or a protein to prevent non-specific antibody binding and reduce background and potentially false positive results.

Blocking with sera or a protein blocking reagent is essential to prevent non-specific binding of antibodies to tissue or Fc receptors (a receptor that binds the constant region (Fc) of an antibody).

A serum matching the species of the secondary antibody is an excellent blocking reagent. Proteins such as bovine serum albumin (BSA) or casein can be used to block non-specific antibody binding.

We recommend blocking endogenous biotin when using an avidin/biotin-based detection system since endogenous biotin is present in many tissues, particularly in the kidney, liver, and brain. You first block before incubating the tissue with avidin and then incubate with biotin to block additional biotin binding sites on the avidin molecule.

If using a primary antibody raised in the same species as your sample (eg mouse antibody on mouse tissue), then block with a F(ab) fragment of a secondary antibody against that species. The F(ab) fragment binds to, and saturates, any endogenous antibodies in the tissue section, blocking binding of the secondary antibody. However, this F(ab) approach does not produce a complete block and does leave some background.

Protein blocking resources

www.abcam.com/normal-serums
www.abcam.com/blocking-reagent-kits

Blocking endogenous enzymes

For enzymatic detection methods, block endogenous enzymes so as not to confound your results. Consider blocking endogenous enzymes after incubating with your primary antibody, as treatments like H_2O_2 can damage epitopes and affect binding. If your antibody is an HRP primary conjugate, then this block needs to be done before the addition of the primary antibody.

Chromogenic detection methods usually use an enzyme conjugated to a secondary antibody to visualize antibody localization. If the enzymatic activity is also endogenous to your tissue sample, it's important to block the endogenous enzymes before the detection step.

Peroxidase blocking

When using horseradish peroxidase (HRP)-conjugated antibodies for detection, non-specific or high background staining may occur due to endogenous peroxidase activity. Incubate tissues with 3,3'-diaminobenzidine (DAB) substrate before primary antibody incubation to check for endogenous peroxidase activity. If the tissues turn brown, endogenous peroxidase (found in red blood cells, for example) is present and you require a blocking step. Hydrogen peroxide (H_2O_2) is the most common peroxidase blocking agent.

Alkaline phosphatase blocking

Endogenous alkaline phosphatase (AP) can produce high background when using an AP-conjugated antibody for detection. Tissue can be tested for endogenous AP by incubating with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride (BCIP/NBT); if a blue color appears, then endogenous AP is present, and blocking is necessary. There are several AP inhibitors available, including levamisole hydrochloride and tetramisole hydrochloride.

The primary antibody

A critical decision when designing an IHC experiment is primary antibody selection since successful immunostaining relies on your primary antibody specifically binding the target antigen.

Direct vs indirect detection

Before choosing your primary, you need to consider whether you plan to use direct or indirect detection methods. The antibody is detected either directly, through a label that is directly conjugated to the primary antibody, or indirectly, using a labeled secondary antibody raised against the host species and antibody type and subtype of the primary antibody.

Direct detection

- Suitable for studying highly expressed antigens
- No need for additional incubation step with a secondary (and therefore eliminates any potential background staining from a secondary)
- Increased flexibility in the design of multi-color experiments

Indirect detection

- Suitable for all antigens
- The signal may be amplified further with various methods (and is discussed in the following sections)
- Requires additional blocking steps and controls

Protocol for immunostaining with paraffin, frozen and free-floating sections

www.abcam.com/IHC-protocol

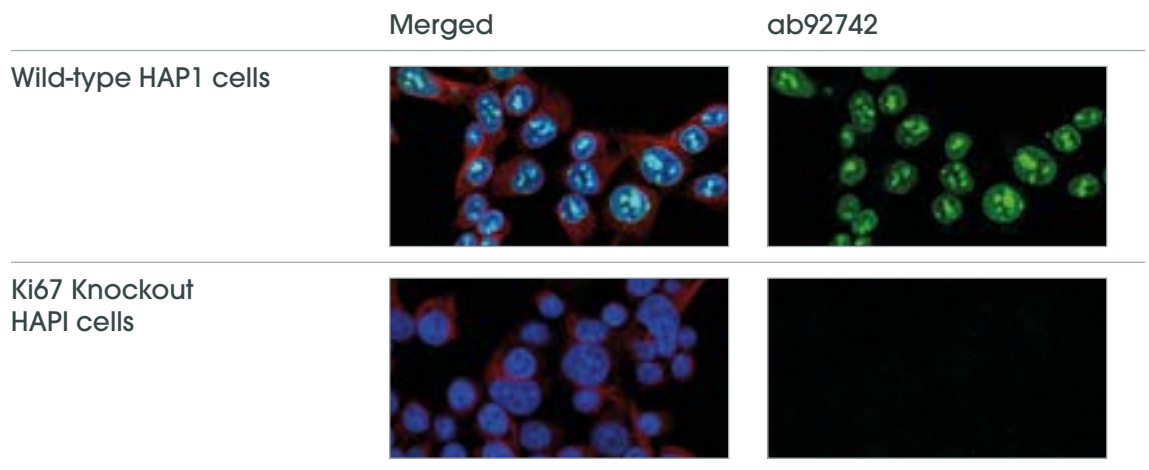
Choosing a primary antibody

Main points to consider: what species is it raised in? Does it bind the intended protein? And has it been shown to work in your application before?

Antibody specificity

The most conclusive demonstration of antibody specificity is lack of staining in tissues or cells in which the target protein has been knocked out. Other indicators are

- Signal-to-noise ratio: the antibody may bind the correct protein, but also have some noise and so you should reject this as having a poor signal-to-noise ratio
 - Particularly relevant if KO is not available and you are determining specificity on localization alone
- Staining patterns that are consistent with known localization of the protein of interest in control cells or tissues
- Lack of staining in tissues or cells known not to express the protein
- Recognition of a single band in western blotting



ICC image of knockout testing for our Ki67 antibody in wildtype (top) and knockout HAP1 cells (bottom). Green is anti-Ki67 (EPR3610) (ab92742) with rabbit IgG (Alexa Fluor® 488) (ab150081), red is anti-alpha-tubulin (Alexa Fluor® 594) (ab195889), blue is nuclear DNA labeled with DAPI.

Prior use in IHC

An antibody that recognizes its target protein in western blotting experiments may not always recognize the antigen in IHC, where the antigen is more likely to be in its native (tertiary 3D) form. An antibody that has been shown to work in IHC is preferable.

Clonality

Antibody clonality is determined by whether the antibodies come from different B-cells (producing polyclonal antibodies) or from identical B-cells derived from a parent clone (producing monoclonal antibodies). These have distinct advantages and limitation.

Table 3. The advantages and limitation of polyclonal vs monoclonal primary antibodies.

	Polyclonal	Monoclonal
Advantages	<ul style="list-style-type: none"> - Potentially a stronger signal as the multiple antibodies bind to the target protein - More tolerant of minor changes in the antigen (eg polymorphism, heterogeneity of glycosylation, or slight denaturation). - More stable over a broader range of pH and buffer compositions. 	<ul style="list-style-type: none"> - Specifically detect a particular or defined epitope on the antigen, making them less likely to cross-react with other proteins. - High degree of homogeneity (especially recombinant antibodies) – if experimental conditions are kept constant, results from monoclonal antibodies can be very reproducible.
Limitations	<ul style="list-style-type: none"> - Prone to a high degree of batch-to-batch variability. - Likely to cross-react and generate non-specific signal*. - Less useful than a monoclonal antibody for probing specific domains on an antigen. 	<ul style="list-style-type: none"> - The epitope targeted by monoclonal antibodies may not be shared across a range of species, limiting their flexibility. - More vulnerable to the loss of epitope through chemical treatment of the antigen than polyclonal antibodies. - Sensitive to changes in experimental conditions (ie pH and buffer composition).

*Antigen/epitope affinity purification makes polyclonal antibodies more specific as a population, especially if the antigen is short, such as a peptide.

Detection and amplification systems

For indirect detection, the secondary antibody is critical to successfully visualizing the distribution of your primary antibody.

Unlike direct detection using a labeled primary antibody, the use of secondary antibodies and related detection systems enable signal amplification as more than one secondary antibody molecule binds to each primary.

Chromogenic vs fluorescent detection

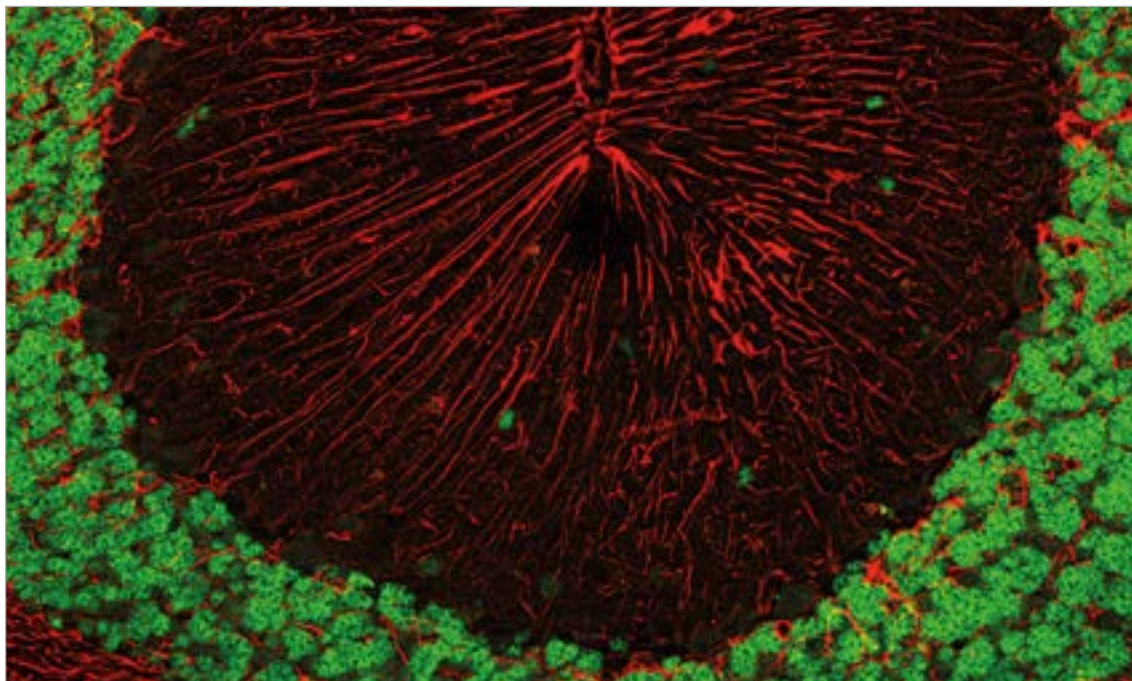
Your detection methods can be either chromogenic, using secondary antibodies that are enzyme-labeled (eg, HRP, AP), or fluorescent (immunofluorescence) using secondary antibodies that are fluorochrome-labeled (eg, FITC, R-PE, Alexa-Fluor®).

Chromogenic

- Biotinylated secondary antibodies and streptavidin-HRP can further amplify the signal in an ABC method. Alternatively, you can use a modern HRP-polymer secondary antibody.
- Some precipitates are photostable (HRP/DAB is very photostable, but HRP/AEC fades in sunlight), potentially allowing storage of the slides for many years.
- Only requires a standard brightfield microscope.
- The enzyme/chromogen precipitate is deposited over a wider area than photons from a fluorescent source, which can affect one's ability to interpret the results.
- The procedure is generally longer as it includes more incubation and blocking steps than fluorescent methods – however, this isn't always the case, depending on which amplification system you use.
- Quantification is generally more difficult due to enzymatic amplification.

Fluorescent

- Useful to visualize multiple antigens simultaneously with multiple fluorophores excited at different wavelengths (multiplexing).
- Better, higher-resolution image quality.
- The signal can be amplified by using HRP antibody labels with tyramide-dyes to deposit fluorescent dyes at the site of antibody staining.
- More amenable to signal quantification.
- Susceptible to auto-fluorescence, particularly with formaldehyde fixation.
- Faster experimental procedure.
- Requires more expensive imaging equipment.
- Fluorophores less stable over time.



Fluorescent IHC image of NeuN in paraffin-embedded mouse cerebellum tissue sections. Green is anti-NeuN (EPR12763) (ab177487), with goat anti-rabbit IgG conjugated to Alexa Fluor® 488 (ab150097), red is anti-GFAP (ab4674), with goat anti-chicken IgY conjugated to Alexa Fluor® 594 (ab150176). Image by Carl Hobbs, Kings's College London, UK.

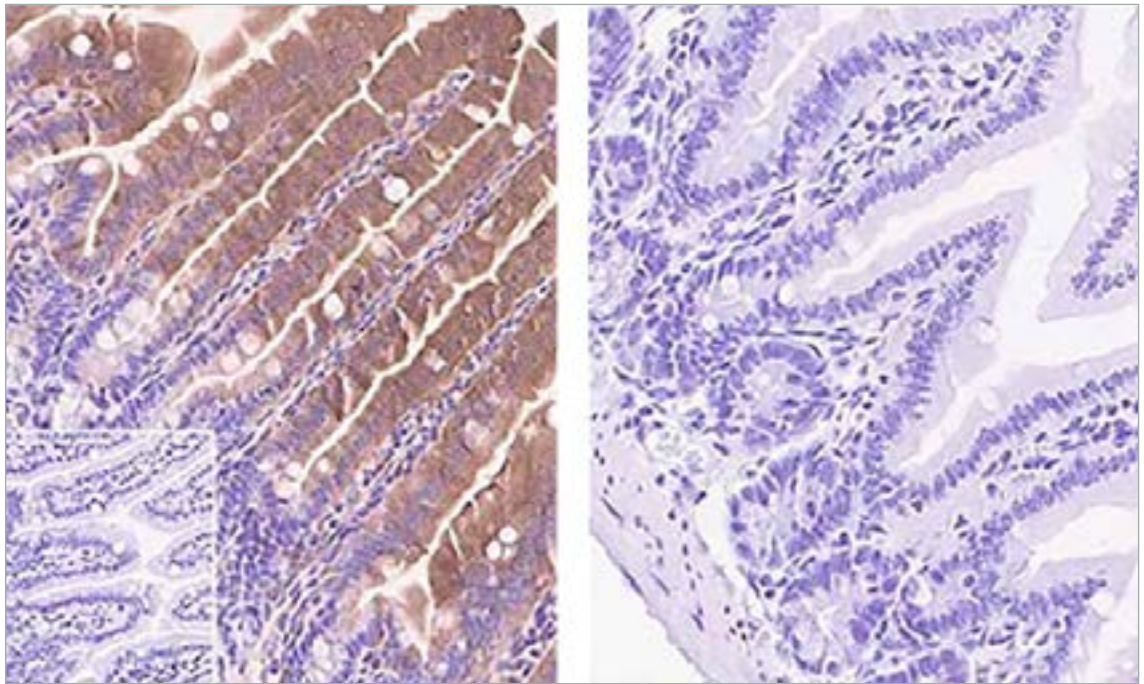
Enzymes and chromogens

Additional factors for consideration in chromogenic detection are the choice of enzymatic and chromogenic substrates. Several different chromogens are available for each detection enzyme (Table 4). HRP-DAB is the most popular combination.

One advantage of chromogens is that you can use them with an organic mounting medium, which tends to have a better refractive index, resulting in sharper images. However, aqueous mediums are faster to use as there is no need to dehydrate the section.

Table 4. Popular enzymes and substrates/chromogens for IHC.

Enzyme	Chromogen/ substrate	Color	Mounting media	Advantages	Disadvantages
HRP	DAB	Brown	Organic/ aqueous	Intense color; permanent	Endogenous peroxidase activity in tissue can lead to false positive staining
	DAB + nickel enhancer	Black	Organic/ aqueous	Intense color; permanent	
	AEC	Red	Aqueous	Intense color; contrasts well with blue in double staining	
AP	BCIP/NBT	Blue/Black	Organic	Intense color	Endogenous AP activity in tissue can lead to false positives
	Fast Red	Red	Aqueous		
	Permanent Red	Red	Organic/ aqueous		



IHC staining of paraffin-embedded wild type (A) and GSDMD KO mouse small intestine (B) with anti-GSDMD antibody (EPR20859) (ab219800) and HRP-polymer conjugated secondary antibody used in our micro-polymer IHC detection kits. Tissue kindly provided by Dr. Feng Shao, NIBS.

Detection and amplification resources

www.abcam.com/IHC-detection-kits
www.abcam.com/secondary-antibodies
www.abcam.com/chromogen-kits

Multi-color IHC

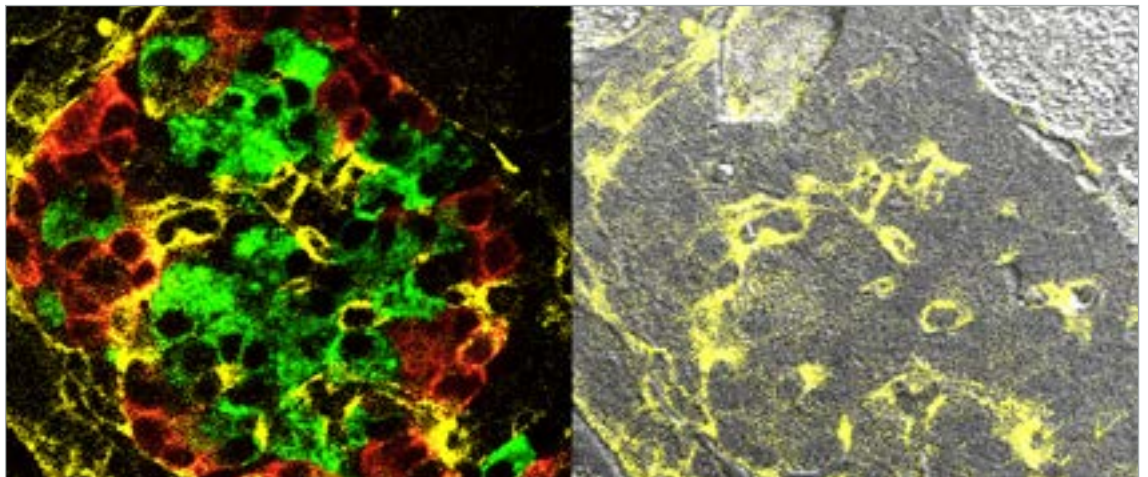
Multiple markers can be immunostained in a single tissue section using multi-color IHC (mIHC).

Traditional chromogenic mIHC relies on each antibody being raised in a different species or of a different isotype. Specific secondary antibodies are then used, with a different chromogen for each marker. However, it is hard to distinguish more than two chromogens on a slide, particularly if any chromogens overlay each other.

Fluorescent mIHC can be easily used with three or more markers. It can be used with fluorescent dye conjugated primary antibodies however it is more commonly used with dye conjugated secondary antibodies, due to their extra amplification, and the limited availability of primary dye conjugates. Most fluorescent mIHC is limited to three markers (plus a counterstain) by available fluorescence filter sets, and by the need for each primary antibody to be raised in a different species / have a different isotype.

The most common methods to increase the number of markers further use: a) spectral unmixing microscopes that enable more fluorescent dyes to be distinguished; and b) sequential antibody stripping and staining methods, often with tyramide signal amplification. Other methods such as imaging mass cytometry, rely on generating a pseudo-image.

mIHC permits high-content data to be generated from one tissue section, effectively reducing the amount of tissue required, and allowing the relationship between different markers to be better understood.



Multi-color fluorescent IHC staining of neonatal pancreas in mice using collagen IV (yellow), insulin (green), and glucagon (red) primary antibodies, and Cy2, Cy5 and Texas Red-conjugated secondary antibodies. Image from Miller K et al. PLoS One 4(11): e7739

Multi-color IHC resources

www.abccam.com/alexa-fluor-secondary-antibodies
www.abccam.com/dye-conjugated-primary-antibodies

Counterstaining

Use a counterstain for specific morphologies or structures to aid localization of your primary antibody.

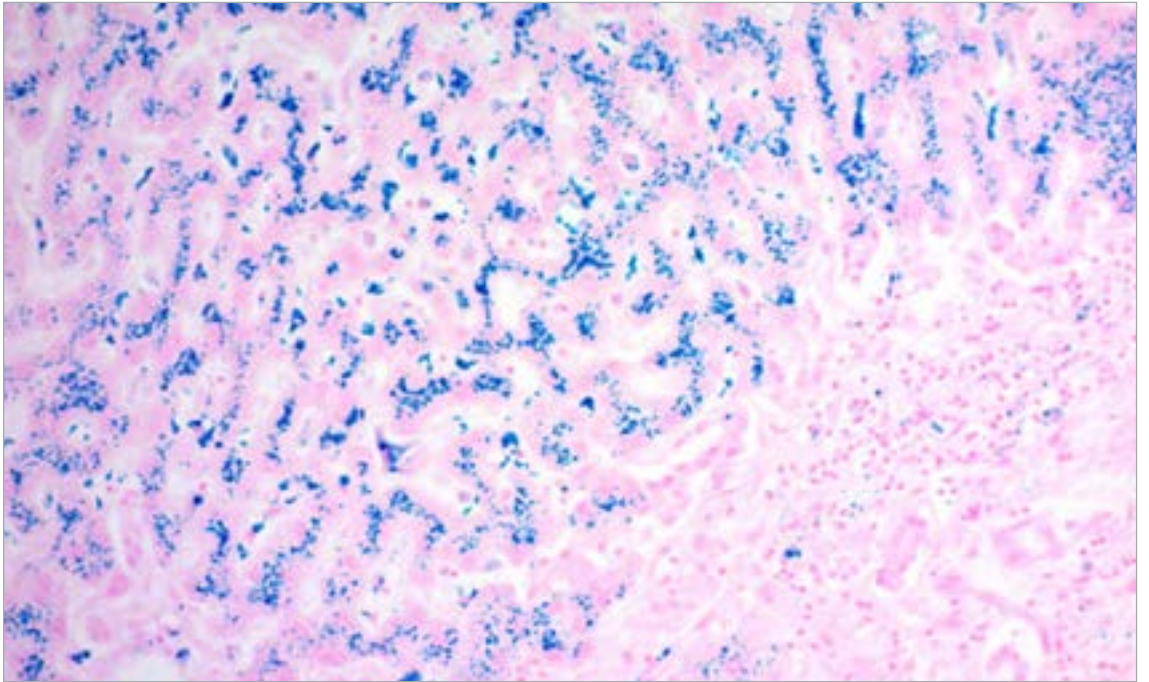
When performing IHC, it is important to use a counterstain, so that you can see where the staining from the antibody is in relation to the cellular structures within the tissue.

The most popular counterstain used with chromogenic IHC staining is hematoxylin, which stains nuclei blue, contrasting with the brown of HRP-DAB. Hematoxylin is 'blued' with a weakly alkaline solution (tap water is sufficient in most areas but this needs to be determined).

In fluorescent IHC the most popular counterstain is the blue nuclear dye DAPI. In both cases, be sure to choose a counterstain that it is compatible with your staining system and doesn't interfere with the signals from your reporter labels.

Table 5. Common counterstains and their targets.

Type	Dye	Target	Color
Chromogenic	Hematoxylin	Nuclei	Blue to violet
Chromogenic	Nuclear fast red (Kernechtrot)	Nucleic acids	Red
Chromogenic	Methyl green	Nucleic acids	Green
Fluorescent	DRAQ5™	Nucleic acids	Red
Fluorescent	DRAQ7™	Nucleic acids	Red
Fluorescent	Nuclear yellow (Hoechst S769121)	Nucleic acids	Yellow/Blue
Fluorescent	Nuclear Green DCS1	Nucleic acids	Green
Fluorescent	Hoechst stain	Nucleic acids	Blue
Fluorescent	DAPI	Nucleic acids	Blue
Fluorescent	Propidium iodide	Nucleic acids	Red



Chemical stains such as Iron Stain Kit (ab150674) (blue: iron stain, pink: nuclear fast red) above can also be used to identify specific features of the tissue and are sometimes used as a counterstain.

Counter-staining resources

www.abcam.com/counterstains-and-special-stains

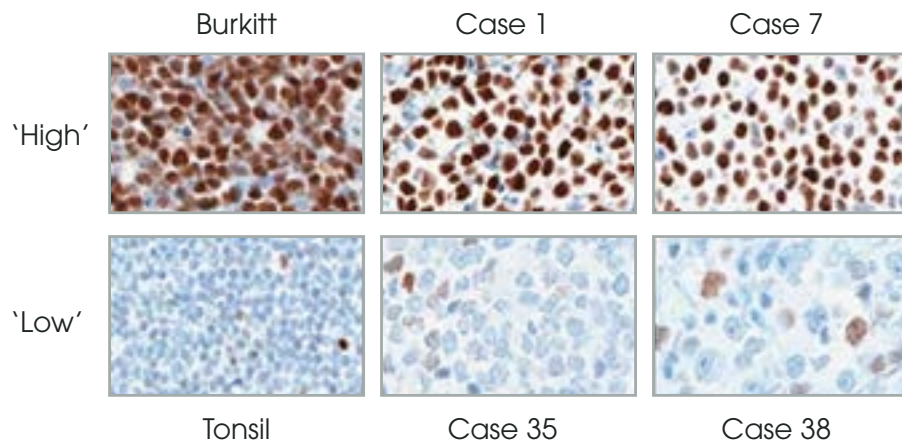
Controls for IHC

Run proper controls so that you can confirm the validity of your staining pattern and exclude experimental artefacts.

You should include several different positive and negative controls and maintain a detailed experimental record to ensure consistent performance.

Antigen (tissue) controls

- **Positive control:** a section from a tissue known to express the protein of interest.
- **Negative control:** a section from a tissue known not to express the target antigen.
- **Endogenous tissue background control:** a section from the tissue that doesn't have primary antibody applied. Certain tissues have inherent properties that result in background staining, which could affect the interpretation of results. For example, certain tissues contain endogenous fluorescent molecules that could be confused for positive staining during fluorescent IHC. The tissue should be checked under the microscope to ensure that there is no endogenous background.



IHC staining of select tumors and reactive tissue stained for c-Myc with anti-c-Myc antibody (Y69) (ab32072) from Kluk MJ et al. PLoS One (2012) 7(4): e33813. Burkitt lymphoma with a confirmed MYC translocation is positive control (high), negative control (low) is healthy tonsil.

Reagent controls

- **No primary antibody control:** tissue is incubated with the antibody diluent alone and no primary antibody, followed by incubation with secondary antibodies and detection reagents.
- **Isotype control:** tissue is incubated with the antibody diluent and a non-immune antibody of the same isotype and at the same concentration as the primary antibody, followed by incubation with the secondary antibodies and detection reagents. This control checks that the observed staining is not caused by non-specific interactions of the antibody with the tissue.
- **Positive reagent control:** tissue incubated with an antibody that detects a ubiquitous target that is known to work with the staining system being employed (including antigen retrieval). This controls to ensures that the methodology and reagents are working as intended.

Troubleshooting IHC experiments

Here are some of the common problems you are likely to encounter during IHC and possible causes and solutions.

No staining

The primary antibody and the secondary antibody are not compatible.

- Use a secondary antibody that was raised against the species in which the primary was raised (e.g., if primary is raised in rabbit, use an anti-rabbit secondary). Check that the isotype of the primary is recognized by the secondary.

Not enough primary antibody is bound to the protein of interest.

- Use a higher concentration of antibody.
- Incubate longer (e.g., overnight) at 4°C.

The antibody may not be suitable for IHC procedures as it may not recognize the native (3D) form of the protein.

- Check the antibody specifications to see if it has been tested in IHC, and what type of IHC (formalin/PFA fixation, fresh frozen etc.). Successful use of the antibody in ICC or IP is also a good indication that the antibody recognizes the native form of the protein.
- Test the antibody in a native (non-denatured) WB to make sure it is still functional.

The primary antibody / secondary antibody / amplification kit may have lost its activity due to improper storage, improper dilution or multiple cycles of freeze/thaw.

- Run positive controls to ensure that these reagents are working properly.

The protein is not present in the tissue of interest.

- Run a positive control recommended in the literature or by the supplier of the antibody.

The protein of interest is not abundant in the tissue.

- Use an amplification step to maximize the signal.

The secondary antibody was not stored in the dark (when performing fluorescence detection).

- Always protect the secondary antibody from exposure to light.

Deparaffinization may be insufficient.

- Deparaffinize sections longer and use fresh xylene.

Fixation procedures may be modifying the epitope that the antibody recognizes.

- Use different antigen retrieval methods to unmask the epitope (heat-mediated with pH 6 or pH 9 buffer, enzymatic, etc.).
- Fix the sections for a shorter time.

The antibody cannot penetrate the nucleus, where the protein is located (nuclear protein).

- Add a strong permeabilizing agent like Triton™ X-100 to the blocking buffer and antibody dilution buffer.

The PBS buffer is contaminated with bacteria that damage the phosphate groups on the protein of interest (phosphorylated proteins)

- Add 0.01% azide to the PBS antibody storage buffer or use fresh sterile PBS.

High background

Blocking of non-specific binding might be absent or insufficient.

- Increase the incubation period with the blocking agent and consider changing blocking agent. If using serum, we recommend 10% normal serum of the species of the secondary antibody for 1 hr. Alternatively, try a commercial blocking buffer or a secondary antibody that has been pre-adsorbed against the immunoglobulin of the species of the samples.

The primary antibody concentration may be too high.

- Titrate the antibody to the optimal concentration, dilute the antibody further and incubate at 4°C.

Incubation temperature may be too high.

- Incubate sections at 4°C.

The secondary antibody may be binding non-specifically.

- Run a secondary control without primary antibody.
- If staining is observed with the secondary alone, change the secondary or use a secondary antibody that has been pre-adsorbed against the immunoglobulin of the species of your samples.

Tissue has not been washed enough; fixative still present.

- Wash extensively in PBS/TBS between all steps. Add a surfactant, such as 0.1% Triton.

Endogenous peroxidases are active.

- Use enzyme inhibitors i.e., levamisol (2 mM) for AP or H₂O₂ (0.3% v/v) for peroxidase.

Fixation procedures are causing autofluorescence (if using fluorescence detection).

- Formalin/PFA usually autofluoresce in the green spectrum, so try a fluorophore in the red range.
- Use a fluorophore in the infrared range if an infrared detection system is available.

Too much amplification (indirect technique).

- Reduce amplification incubation time and dilute the secondary antibody or amplification reagent.

Too much substrate was applied (enzymatic detection).

- Dilute substrate further or reduce substrate incubation time.

The chromogen reacts with PBS present in the tissue sample (enzymatic detection).

- Use Tris buffer to wash sections before incubating with the substrate, then wash sections/cells in Tris buffer (NB, this only applies to AP).

Permeabilization has damaged the membrane and removed the membrane protein (membrane protein).

- Use a less stringent detergent (e.g., Tween[®] 20 instead of Triton[™] X-100).
- Remove permeabilizing agent from your buffers.

Non-specific staining

Primary/secondary antibody concentration may be too high.

- Try decreasing the antibody concentration and/or the incubation period. Compare signal intensity against tissue that does not express the target protein.

Endogenous peroxidases are active.

- Use enzyme inhibitors i.e., levamisol (2 mM) for AP or H₂O₂ (0.3% v/v) for peroxidase.

The primary antibody is raised against the same species as the tissue stained (e.g., mouse primary antibody tested on mouse tissue). When the secondary antibody is applied, it binds to all the tissue as it is raised against that species.

- Use a primary antibody raised in a different species than your tissue, or block with a F(ab) fragment secondary antibody.

The sections/cells have dried out.

- Keep sections/cells at high humidity and do not let them dry out.

Poorly resolved or damaged tissue morphology

Antigen retrieval methods may be too harsh.

- Vary antigen retrieval procedure or try different antigen retrieval methods.

The tissue may have been underfixed.

- Increase fixation time.
- Increase ratio of fixative to tissue.
- Cut smaller pieces of tissue for more efficient fixation (fixation by immersion).

Tissue sections are falling off the slide (frozen sections).

- Increase fixation time.
- Try an alternative fixative.
- Use freshly prepared slides.

Tissue sections are torn or folded, or air bubbles are visible under the sections.

- Re-cut sections using a sharp blade.
- Study areas of tissue that are unaffected. Localize reagents using a PAP pen (ab2601).

The tissue morphology is poorly resolved.

- Cut thinner tissue sections.
- Ice crystals may have destroyed morphology of sections - re-cut and freeze rapidly (frozen).

Tissue has autolysed.

- Increase fixation time.
- Increase ratio of fixative to tissue.
- Try using cross-linking fixative.

