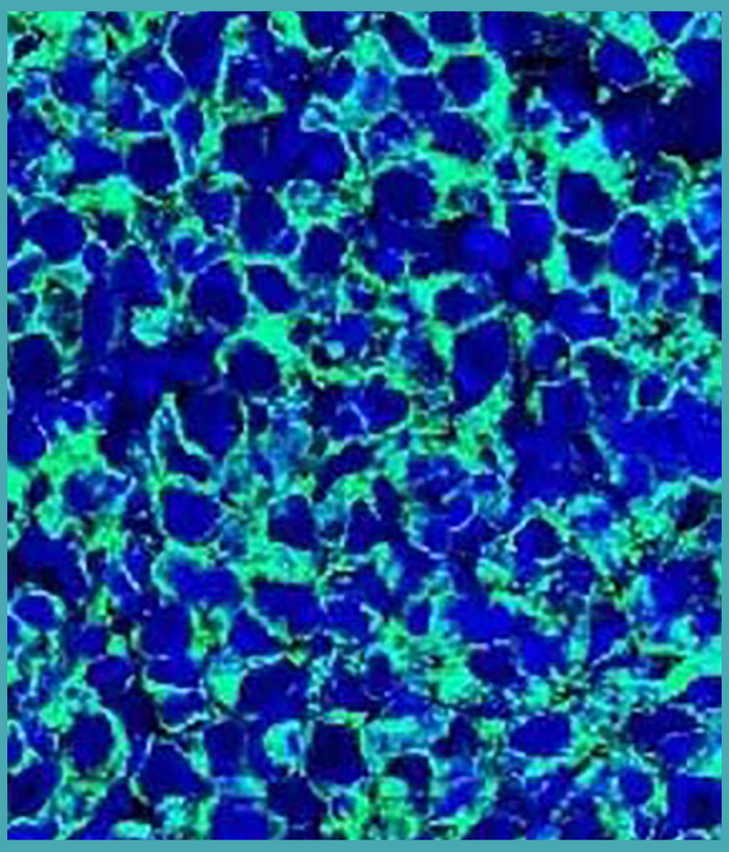
The complete guide to immunohistochemistry



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Contents

What is IHC?

Immunohistochemistry is a powerful tool for visualizing the distribution and localization of antigens in tissue sections.

Immunohistochemistry (IHC) uses antibodies to detect the location of proteins and other antigens in tissue sections. Since antibodies are highly specific, the antibody will bind only to the antigen of interest. The antibody-antigen interaction is then viewed using either a colored enzyme substrate (chromogenic detection) or a fluorescent dye (fluorescent detection).

Although IHC is less quantitative than western blotting or ELISA, it gives valuable information about protein localization. Protein expression patterns are tremendously beneficial in research and healthcare and can be an important diagnostic tool to determine abnormal cells in diseases such as cancer.

Sample preparation

Sample preparation is key to producing high-quality staining during immunohistochem istry (IHC).

Sample preparation for IHC may include fixation, dehydration, embedding, and sectioning, but one or two experimental variables usually determine the exact workflow.

Additional steps in IHC sample preparation may include antigen retrieval to unmask any epitopes altered by fixation, permeabilization to grant the antibody access to intracellular proteins, and blocking to prevent non-specific staining.

Tissue fixation in IHC

Fixation of the tissue sample is essential to preserve tissue morphology and retain the antigenicity of the target protein during the IHC experiment and storage. Fixation can also enhance the refractive index of tissue constituents and give tissue support during sectioning.

The choice of fixative depends on the target antigen and the desired detection technique (fluorescent or chromogenic). Furthermore, the fixation method often drives the design of the sample preparation workflow; for example, tissues may need to be snap-frozen if a phosphorylated epitope is being studied. Fixation usually occurs before embedding in paraffin but after freezing.

Using paraffin-embedded samples in IHC

When generating paraffin-embedded tissue samples, the tissue must be fixed before embedding in paraffin. Fixation is achieved by perfusion or immersion immediately after dissection and typically takes 4 - 24 hours. Fixation for longer than 24 hours is not recommended as it may lead to over-fixation, which may mask the antigen.

Standardized fixatives for each type of antigen are essential for reproducible staining, as an antigen that has been inappropriately fixed may not be detected. The most suitable fixative for an IHC experiment depends on the antigen, as illustrated in the figure below. Some guidelines for the type of fixative to use are given in Table 1.

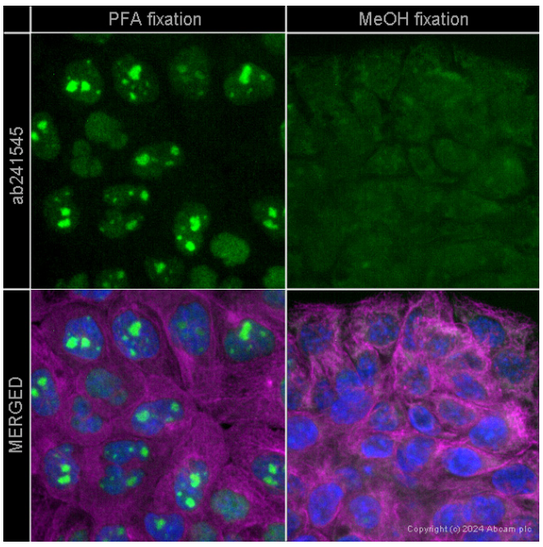


Figure 1. Effect of fixative on immunostaining patterns - the target protein Werner's syndrome helicase WRN is detected in immunocytochemistry of A431 cells fixed with PFA (left) and shows the expected nuclear localization. However, the localization in MeOH fixed cells is weak in the nucleus and cytoplasm indicating non-specific binding. Primary: ab241545, Mouse monoclonal [195C] to Werner's syndrome helicase WRN, 5 µg/ml. Secondary: ab150117, goat anti-mouse IgG H&L (Alexa Fluor® 488), 2 µg/mL (shown in green). Nuclei are stained with DAPI (shown in blue) and tubulin is stained with ab6046 and ab150080 (shown in magenta).

After fixation, the tissue is dehydrated to enable embedding with paraffin, which is water-insoluble. The tissue is dehydrated gently by immersion in increasing concentrations of a dehydrating agent such as alcohol. This gradual change in hydrophobicity minimizes cell damage.

Clear the dehydrating agent by incubation in xylene before paraffin embedding. Paraffin is typically heated to 60°C and then allowed to harden overnight. Finally, section the tissue using a microtome.

Tissue sections may be dried onto microscope slides and stored for extended periods at room temperature. Rehydrate the tissue sections before commencing the immunostaining protocol.

Using frozen samples in IHC

Prepare frozen samples by immersing the tissue in liquid nitrogen, isopentane, or burying it in dry ice. Snap-freezing is frequently used when detecting post-translational modifications such as phosphorylation.

After freezing, cut the tissue using a cryostat, and the resulting sections can be stored at -80°C for up to one year. Frozen tissue sections are typically fixed with an alcohol such as methanol or ethanol. As alcohols do not mask epitopes, their use avoids the need for antigen retrieval.

What is the difference between formaldehyde and formalin?

Formaldehyde is a gas that retains its chemical properties in an aqueous solution, whereas formalin is a saturated solution of 37-40% w/v formaldehyde in water. 10% formalin is, therefore, roughly equivalent to 4% formaldehyde.

Formalin contains ~10% methanol, which the manufacturer adds to slow the polymerization of formaldehyde in solution to paraformaldehyde. Therefore, paraformaldehyde is a solid comprised of large polymers of formaldehyde.

As the added methanol can negatively impact the fixation of certain samples, some protocols recommend making formalin from paraformaldehyde immediately before sample fixation.

Table 1. Guidelines for choosing a fixative

Antigen	Recommended fixative
Most proteins, peptides and enzymes of low molecular weight	Cells / cytological preparations: 4% formaldehyde Tissue sections: 10% Neutral-Buffered Formalin (NBF)
Delicate tissue	Bouin's fixative
Small molecules such as amino acids	4% formaldehyde
Blood-forming organs (liver, spleen, bone marrow)	Zenker's solution
Connective tissue	Helly's solution
Nucleic acids	Carnoy's solution
Large protein antigens (e.g., immunoglobulin)	Ice-cold acetone or methanol (100%)
Nuclear morphology	Zinc formalin
For electron microscopy	4% formaldehyde - 1% glutaraldehyde

Antigen retrieval and permeabilization for IHC

After sample preparation, two further steps may be required: antigen retrieval and permeabilization.

Antigen Retrieval

The process of sample fixation can sometimes lead to protein cross-linking, which masks antigens and can restrict antigen-antibody binding. Antigen retrieval enables an antibody to access the target protein within the tissue. This step is not necessary if the fixation was mild. For example, if frozen tissues were fixed with alcohol, antigen retrieval isn't required as alcohols do not mask epitopes.

Masked epitopes can be recovered using either enzymatic/proteolytic antigen retrieval (PIER) or heat-induced antigen retrieval methods (HIER). Whereas PIER uses proteases, such as proteinase K, trypsin, and pepsin, HIER uses heat (from either a microwave, pressure cooker, steamer, water bath, or autoclave) and a selection of buffers. For a comparison of HIER and PIER, see Table 2 below.

The optimal antigen retrieval technique is dependent on several factors, including the antigen, tissue, fixation method, and primary antibody. Some antigens require a combination of heating and enzyme digestion. To identify the best method for your specific antigen, we recommend testing two methods of HIER, for example, citrate buffer pH 6 and Tris-EDTA pH 9, and one or two methods of PIER, for example, proteinase K and trypsin.

Table 2. Comparison of HIER and PIER antigen retrieval methods

	HIER	PIER
Advantages	Gentler epitope retrieval and more definable parameters	Useful for epitopes that are difficult to retrieve
pH	Citrate buffers of pH 6 are often used but high pH buffers have been shown to be widely applicable for many antibodies. Optimal pH must be determined experimentally.	Typically, pH 7.4
Temperature	Approximately 95˚C	Typically, 37˚C
Incubation time	10-20 minutes (commonly, 20 minutes)	5-30 minutes (commonly, 10-15 minutes)
Buffer composition	Depends on pH required (pH is target-dependent). Popular buffers include sodium citrate, EDTA and Tris-EDTA	Neutral buffer solutions of enzymes such as pepsin, proteinase K or trypsin
Precautions	Heating using a microwave can result in unbalanced epitope retrieval due to uneven heating. Boiling can also lift tissue off of the slide.	Excessive enzymatic retrieval sometimes damages tissue morphology

Permeabilization for IHC

Permeabilization is required for the antibody to access the inside of cells to detect the target antigen. Such antigens include intracellular proteins and cytoplasmic epitopes of transmembrane proteins.

Solvents or detergents are typically used for permeabilization. Solvents are generally recommended for cytoskeletal, viral, and some enzyme antigens. Detergent permeabilization can significantly improve antibody access to antigens in the cytoplasm, on the cytoplasmic face of the plasma membrane, and soluble nuclear antigens. Depending on your antigen of interest, either harsh or mild detergents can be used. Harsh detergents, such as Triton™ X-100 or NP-40, can disrupt proteins, whereas mild detergents, such as Tween 20®, saponin, or digitonin, do not dissolve plasma membranes.

Table 3. Solvent and detergent guidelines

	Solvents	Comments
Solvents	Acetone	Acetone fixation will also permeabilize
	Methanol	Methanol fixation can be used to permeabilize but is not always effective
Detergents	Triton™ X-100 or NP-40	Use 0.1 to 0.2% in PBS for 10 min only
	Tween 20®, saponin, digitonin and Leucoperm	Use 0.2 to 0.5% for 10 to 30 min

Blocking

Blocking is an essential step in your immunohistochemistry (IHC) experiments as it reduces background signals and false positives.

Blocking occurs after sample preparation but usually before incubation with the primary antibody. As IHC uses antibodies to recognize specific antigens, blocking is crucial to prevent non-specific binding. Without this step, the primary antibody may bind to several sites and cause a false-positive result.

Protein blocking in IHC

Serum is a common protein-blocking agent as it contains antibodies that bind to non-specific sites. For best results, use a serum that matches the secondary antibody species; this prevents the detection of non-specific binding sites (Figure 2). If you are performing multiple stains using secondary antibodies from different species, it may be necessary to use blocking sera from the species of both secondary antibodies.

Other commonly used protein-blocking reagents are bovine serum albumin (BSA) and casein from nonfat dry milk. Pre-formulated blocking buffers are also available, which are often formulated to optimize performance or shelf life. These methods do not need to be matched to the species of the secondary antibody.

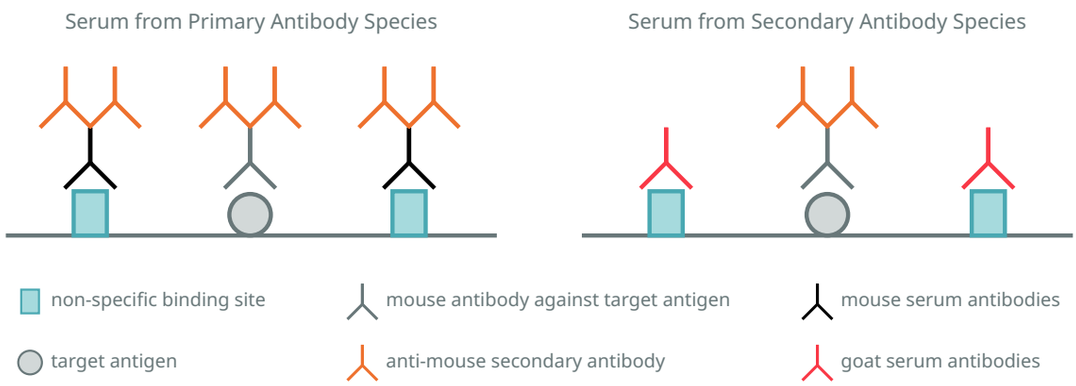


Figure 2. Detection of specific and non-specific binding sites with primary and secondary antibodies

Using mouse antibodies on mouse tissue

Extra care must be taken when using mouse primary antibodies with mouse tissue. High background staining can occur as the anti-mouse secondary antibody binds to endogenous mouse IgG and Fc receptors. This background binding can be reduced using F(ab) fragments, as described in our mouse-on-mouse protocol, and it is important to match the blocking fragment carefully and the secondary antibody used.

Reducing autofluorescence in IHC

If a fluorescent label is used for detection, check if your tissue is autofluorescent, as this can lead to false-positive results and high background. There are several causes of autofluorescence, including the fixation method and fluorescent compounds. However, if endogenous autofluorescence cannot be blocked, a chromogenic detection system may be preferable.

Causes and tips to reduce autofluorescence:

- Tissue fixation, particularly when using aldehyde fixatives (eg formalin), which react with amines to generate fluorescent products. This can be reduced through:
  - Use non-aldehyde fixatives, such as Carnoy's solution
  - Block aldehydes by treating samples with sodium borohydride or glycine/lysine.
  - Use frozen tissue sections
  - Treat tissue with quenching dyes such as pontamine sky blue, Sudan black, trypan blue, or FITC block.
- Fluorescent compounds, such as flavins and porphyrins. The effect of these can be reduced through:
  - Extract these compounds from the tissue by the solvents used to generate fixed, dehydrated sections. However, they persist in frozen sections that have been processed using aqueous reagents.

Blocking endogenous enzymes

Chromogenic detection uses enzymes to visualize the target antigen. Therefore, endogenous enzymes need to be blocked before detection.

Biotin blocking

If you are using a biotin-based detection system, blocking endogenous biotin is recommended. First, an excess of avidin is added to the sample to bind any naturally occurring biotin. This is followed by incubation with an excess of biotin to block additional binding sites on the avidin molecule.

Consider using a polymer-based method if the sample is high in endogenous biotin, such as the kidney, liver, and brain. Polymer methods use a dextran backbone to which multiple enzyme molecules and secondary antibodies are attached.

Peroxidase blocking

Some tissues, including the kidney, liver, and those containing red blood cells (such as vascular tissue), contain endogenous peroxidases. If you are using horseradish peroxidase (HRP) to view proteins of interest, check for endogenous peroxidase activity by incubating the sample in 3,3'-diaminobenzidine (DAB) substrate. If the sample turns brown, endogenous peroxidase is present, and a blocking step is required. A 10-15 minute incubation in 0.3% hydrogen peroxide is usually sufficient blocking.

Alkaline phosphatase blocking

Endogenous alkaline phosphatase (AP) can be found in the kidney, intestine, osteoblasts, lymphoid tissue, and placenta and generally has higher activity in frozen tissue.

If you are using AP for detection, determine if the sample contains endogenous AP by incubation with a BCIP/NBT solution. If the sample turns blue, endogenous AP is present, and blocking is necessary. Blocking is achieved by incubation with an alkaline phosphatase inhibitor, such as levamisole hydrochloride or tetramisole hydrochloride.

Antibodies in IHC

Choosing the correct antibodies is critical to the success of the IHC experiment.

Direct and indirect detection

Successful immunostaining relies on the primary antibody specifically binding to the target antigen. The antibody is then detected either directly, through a label conjugated to the primary antibody, or indirectly, using a labeled secondary antibody.

Direct detection is ideal for detecting highly expressed antigens and doesn't require an additional incubation step with a secondary reagent. Indirect detection is more suitable for studies of poorly expressed antigens, which benefit from the signal amplification provided by the secondary reagent.

Signal amplification occurs through the potential for two or more labeled secondary antibodies to bind to each primary antibody. The signal may be amplified further by using avidin or streptavidin with biotinylated secondary antibodies. However, the use of a secondary antibody requires additional blocking steps and controls.

	Direct	**Indirect**
Advantages	Increased flexibility for multicolor experiments.	Suitable for all antigens.
	No need for additional incubation step.	Signal may be amplified further.
	Removes potential background staining from a secondary antibody.	
Disadvantages	Only suitable for highly expressed antigens.	Requires additional blocking steps and controls

Choosing and optimizing primary antibodies for IHC

As the choice of primary antibody is critical to the success of the IHC experiment, it is essential to consider these factors.

Is the antibody specific for the protein/epitope of interest?

The specificity of the antibody is usually determined experimentally. Staining patterns consistent with the known localization of the target protein in control cells or tissues can indicate specificity.

Comparison of the immunogen sequence to other proteins using alignment tools such as BLAST may also indicate antibody specificity, but it is not conclusive.

The most definitive demonstration of antibody specificity is the lack of staining in tissues or cells where the target protein has been knocked out.

Has the antibody been proven to work in IHC?

Antibodies are validated for distinct techniques due to differences in antigen recognition. For example, western blotting experiments are run under denaturing conditions, whereas the native (3D) form of the protein is more likely to be preserved in IHC. Consequently, an antibody that has been validated for western blot may not work in IHC.

It is important to note that fixation and antigen retrieval methods also significantly impact the ability of an antibody to recognize the epitope of interest in an IHC experiment.

The method of antibody manufacture

There are three main methods of antibody manufacture – serum-purified polyclonal, hybridoma-derived monoclonal, and recombinant. The type of antibody manufacture can significantly impact your results, so it is essential to understand the different methods:

— Serum-purified polyclonal antibodies are a heterogeneous mix of antibodies obtained by immunizing an animal with the antigen of interest.

— Hybridoma-derived monoclonal antibodies are produced from a single-clone antibody-producing B-cell that is fused to a myeloma.

— Recombinantly manufactured antibodies are produced recombinantly by cloning antibody-coding genes into high-yield expression vectors.

Which host species should be used for the primary antibody?

Ideally, the primary antibody should be raised in a host species different from the sample species to avoid cross-reactivity with endogenous immunoglobulins in the tissue. If you are using a mouse primary antibody on mouse tissue, specialized mouse-on-mouse IHC kits are available.

Antibody optimization

The quality of staining is influenced by many variables, including the primary antibody concentration, the diluent used, the incubation time, and temperature. Optimization may be required for each antibody and sample in order to achieve specific staining with minimal background. To gain optimal staining:

— Vary antibody concentration while maintaining a constant incubation time and temperature.

— Try a longer incubation time to help the antibody penetrate the tissue

— Combine longer incubation times with lower temperatures to promote specific binding, eg instead of 1 h at room temperature, try overnight at 4°C.

Secondary antibodies in IHC

To ameliorate non-specific binding, the secondary antibody must be directed at the species in which the primary antibody was raised but must itself be raised in a different species.

For example, if the primary antibody is raised in rabbit, an anti-rabbit secondary antibody raised in a species other than rabbit must be used. It is also important that the isotype that the secondary antibody is raised against matches the primary antibody's isotype.

As with primary antibodies, there are considerations for the optimal secondary antibody.

Pre-adsorption of secondary antibodies

Pre-adsorption is an extra purification step to increase the specificity of a secondary antibody. The secondary antibody is passed through a column matrix containing immobilized serum proteins to separate the highly specific antibodies from the non-specific antibodies.

$F(ab')_2$ fragment secondary antibodies

$F(ab')_2$ fragment secondary antibodies are recommended for staining tissues that are rich in Fc receptors (eg spleen, thymus, blood). $F(ab')_2$ fragment secondary antibodies, which are smaller and penetrate tissues more efficiently, are beneficial for multiple IHC staining.

HRP-polymer secondary antibodies for low-expressing proteins

For higher sensitivity, horseradish peroxidase (HRP)-polymer secondary antibodies use micropolymer technology to form smaller detection complexes that allow improved tissue penetration and sensitivity. In addition, HRP-polymer secondaries bind more HRP than standard HRP secondary antibodies, increasing signal.

Detection and amplification systems

In IHC, antigens can be detected by either chromogenic or fluorescent methods.

Chromogenic detection relies on enzymes that convert soluble substrates into insoluble chromogenic products. Alternatively, fluorescent detection uses fluorochrome labels, which emit light of a longer wavelength when excited by light of a specific wavelength.

Chromogenic detection in IHC

The enzymes used to detect antigens in chromogenic detection are typically conjugated to secondary antibodies, although primary antibodies directly conjugated to the enzyme can also be used.

The most commonly used enzymes are horseradish peroxidase (HRP), which converts 3,3'-diaminobenzidine (DAB) into a brown product, and alkaline phosphatase (AP), which converts 3-amino-9-ethyl carbazole (AEC) into a red product. However, the choice of chromogen is determined by the enzyme used.

Table 4. View guidelines for choosing which chromogen to use

Enzyme	Chromogen	Color	Mounting media	Advantages (+) and disadvantages (-)
Horseradish peroxidase (HRP)	AEC	Red	Aqueous	+ Intense color, contrasts well with blue in double staining.
Horseradish peroxidase (HRP)	DAB	Brown	Organic	+ Intense color; permanent.
Horseradish peroxidase (HRP)	DAB + nickel enhancer	Black	Organic	+ Intense color; permanent.
Horseradish peroxidase (HRP)	TMB	Blue	Aqueous	+ Intense color; permanent.
Horseradish peroxidase (HRP)	StayYellow	Yellow	Aqueous	+ Alternate color useful in multicolor chromogenic IHC
Alkaline phosphatase (AP)	BCIP/NBT	Blue	Organic	+ Intense color.
Alkaline phosphatase (AP)	BCIP/TNBT	Purple	Organic	+ Intense color.
Alkaline phosphatase (AP)	Naphthol AS-MX phosphate + Fast Blue BB	Blue	Aqueous	+ Less intense, good for double staining. - Fast Blue BB prone to fading.
Alkaline phosphatase (AP)	Naphthol AS-MX phosphate + Fast Red TR	Red	Aqueous	+ Less intense, good for double staining. - Fast Red TR prone to fading.
Alkaline phosphatase (AP)	Naphthol AS-MX phosphate + new fuchsin	Red	Organic	+ Intense color. - Fast Red prone to fading.

Alkaline phosphatase (AP)	StayGreen	Green	Organic	+ Alternate color useful in multicolor chromogenic IHC
Glucose oxidase	NBT	Blue	Organic	+ No endogenous enzyme activity, so does not suffer from endogenous peroxidases causing false positive staining. - Low staining intensity. High antibody concentrations needed.

Four principal methods of indirect chromogenic detection are widely used. The biotin-based methods use an avidin-biotin complex (ABC) or a labeled streptavidin-biotin binding (LSAB) complex. The non-biotin based methods employ a polymer complex or a micropolymer complex.

Avidin-biotin complex (ABC) method

Early use of the ABC method relied on biotinylated secondary antibodies and an avidin-biotin- reporter enzyme complex. As avidin is tetravalent, large complexes form, resulting in high signal intensity.

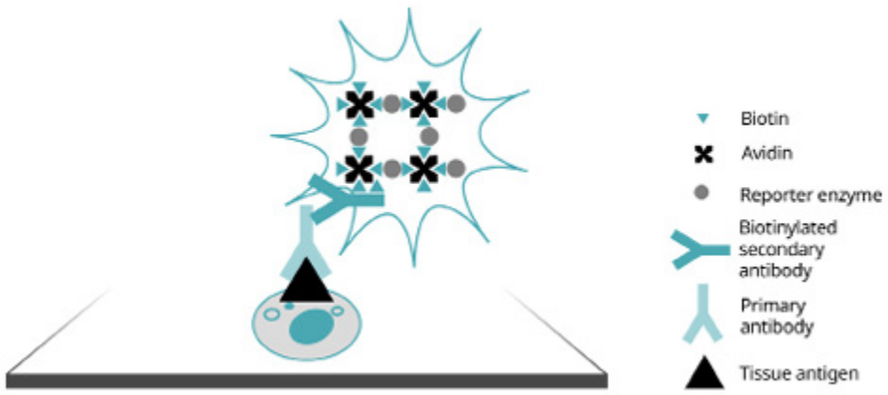


Figure 3. Antibody detection via avidin-biotin complex method

Labeled streptavidin-biotin (LSAB) method

Most detection now relies on the LSAB variant of the ABC method, which uses streptavidin, instead of avidin. This results in less non-specific tissue binding, as streptavidin is not glycosylated and has a more neutral isoelectric point than avidin.

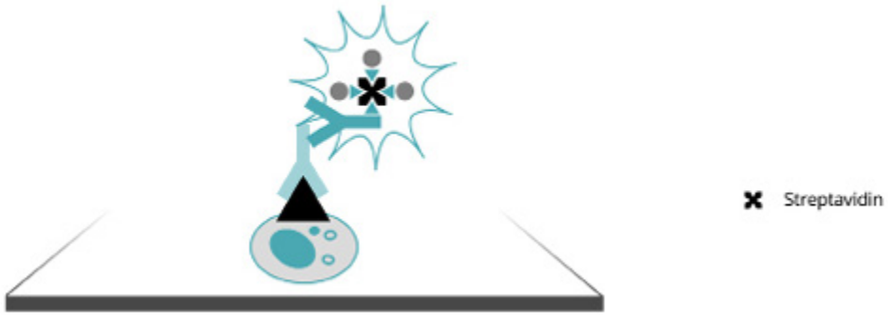


Figure 4. Antibody detection via streptavidin-biotin method

Polymer methods

The key challenge of biotin-based systems is that endogenous biotin can lead to significant background staining in certain tissues (eg brain). While formalin fixation and paraffin embedding reduce biotin levels, antigen retrieval can expose biotin.

In frozen sections, endogenous biotin is a significant problem. Although extra steps with biotin blocking solutions can be used to reduce background, non-biotin polymer-based methods offer an alternative.

Early polymer methods used a dextran backbone to which multiple enzyme molecules and secondary antibodies are attached. More recent micro-polymer / compact polymer methods use a smaller detection complex with less tendency to aggregate. This results in greater sensitivity through better tissue penetration and reduced background staining from endogenous biotin.

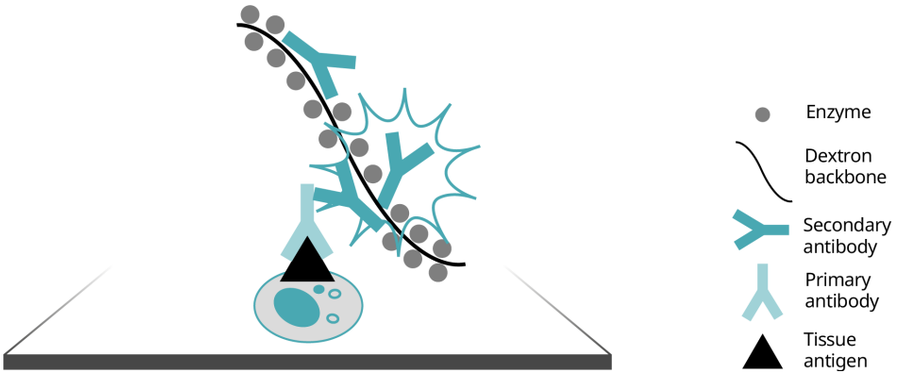


Figure 5. Antibody detection via dextran backbone

Counterstains and special stains for IHC

Histology, fluorescent, and special stains are used to stain specific cell and tissue structures and microorganisms to help identify the location of primary antibody staining.

Chromogenic counterstains are used when the primary antibody is visualized using HRP or alkaline phosphatase combined with DAB, AEC, or similar enzyme substrates. The traditional combination is HRP/DAB with hematoxylin to stain nuclei. Eosin, for cytoplasmic staining, is often used in combination with hematoxylin.

Fluorescent counterstains are used when the primary antibody staining is visualized with a fluorescent dye conjugated directly to the primary or secondary antibody. You should avoid using a counterstain with an overlapping emission spectrum to the dye used for primary antibody visualization. DAPI is traditionally the most popular fluorescent nuclear counterstain.

Special stains are used to stain cell types, microorganisms, and specific proteins, carbohydrates, and metabolites found in the tissue matrix and within cells. While simple to use once established, special stains are often time-consuming to set up and optimize. Optimized special stain kits provide a convenient alternative to setting up a stain yourself, and they are often faster to use than the traditional staining methods.

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

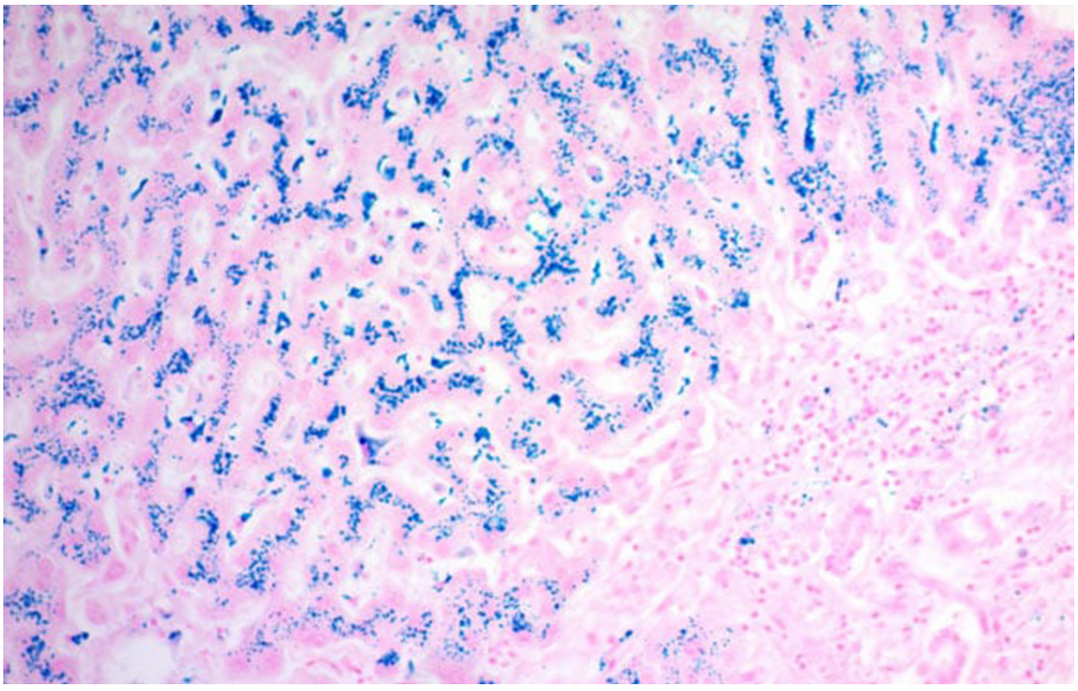


Figure 6. Example of the use of special stains and counterstains. Prussian blue iron stain kit staining iron (blue) in liver tissue. Nuclei and background were counterstained with nuclear fast red.

Advantages of chromogenic detection over fluorescent detection

Chromogenic detection is usually more sensitive than fluorescent detection due to the higher signal amplification. Furthermore, unlike fluorophores, the colored precipitates created from substrates such as DAB are photostable, enabling storage of the slides for many years.

While fluorescent detection requires specialized light sources and filters, chromogenic detection only requires a standard microscope. However, the experimental procedure is longer as it includes more incubation and blocking steps than fluorescent methods.

Multicolor and fluorescent detection in IHC

Staining up to three antigens simultaneously using chromogens is possible, which typically requires primary antibodies raised in different species. If the antigen is present at a high enough level, a primary antibody directly conjugated to the reporter enzyme can be used. If you are using two primary antibodies raised in the same species, binding sites on the secondary antibody must be blocked before staining with the second primary antibody.

Alternatively, fluorescent detection is widely used to visualize multiple antigens simultaneously. The fluorochrome may be conjugated directly to the primary or secondary antibody or streptavidin.

When designing multicolor experiments, two key parameters must be considered:

— The spectral overlap between the fluorochromes being used should be limited as much as possible.

— If indirect detection methods are employed, cross-reactivity between the detection reagents must be avoided where possible. This is usually achieved by selecting primary antibodies from different species, ensuring that each secondary antibody only recognizes one primary antibody in the experiment.

Two primary antibodies from the same species may be used if one of the primary antibodies is biotinylated. The tissue is first incubated with the non-biotinylated antibody and the corresponding fluorochrome-conjugated secondary antibody. The tissue is then incubated with the biotinylated antibody, followed by a streptavidin-conjugated fluorophore. This method is susceptible to high background staining due to endogenous biotin, particularly when using frozen tissues.

Mounting media

After staining, a mounting medium is used to adhere a coverslip to a tissue section or cell smear. Mounting the tissue specimen is essential for preservation during storage and enhancing imaging quality during microscopy.

There are two categories of mounting media, organic and aqueous (or hydrophobic and hydrophilic, respectively). Organic mounting media can only be used for enzymatic labels where the precipitate formed between the enzyme and the chromogen is not soluble in the organic solvents used during mounting of the tissue (eg diaminobenzidine, DAB).

Aqueous mounting media are generally suitable for all enzymatic label/chromogen combinations and fluorescent labels. If you are using fluorescent labels, use a mounting media that preserves fluorescence to enable imaging of samples after prolonged storage.

Recommended mounting media for non-fluorescent imaging

Product name	Type
Limonene Mounting Medium	Organic
Mounting Medium for IHC	Aqueous

Recommended mounting media for fluorescent imaging

Product name	Type
Anti-Fade Mounting Medium	Aqueous
Anti-Fade Mounting Medium With DAPI	Aqueous
Anti-Fade Mounting Medium With PI	Aqueous

Controls in IHC

It is essential to run controls in IHC staining experiments to confirm that the observed staining pattern is accurate and reliable.

Antigen (tissue) controls

Positive control

A positive control is known to express the protein of interest. Even if the samples are negative, positive results from the positive control will indicate that the procedure is working and optimized, verifying that any negative results are valid.

Negative control

A negative control is known not to express the target antigen. This is to check for non-specific signals and false-positive results.

Endogenous tissue background control

An endogenous tissue background control is a section from the tissue before applying the primary antibody. Certain tissues have inherent properties that result in background staining, affecting the interpretation of results. For example, tissues containing endogenous fluorescent molecules could be confused for positive staining during fluorescent IHC. The tissue should be checked under the microscope using either fluorescent or bright-field illumination (for fluorescent or chromogenic labels, respectively) to ensure no endogenous background.

Reagent controls

No primary antibody control

A no primary antibody control confirms the staining is produced from detection of the antigen by the primary antibody and not by the detection system or the specimen. This involves incubating the sample with the antibody diluent alone and no primary antibody, followed by incubation with secondary antibodies and detection reagents.

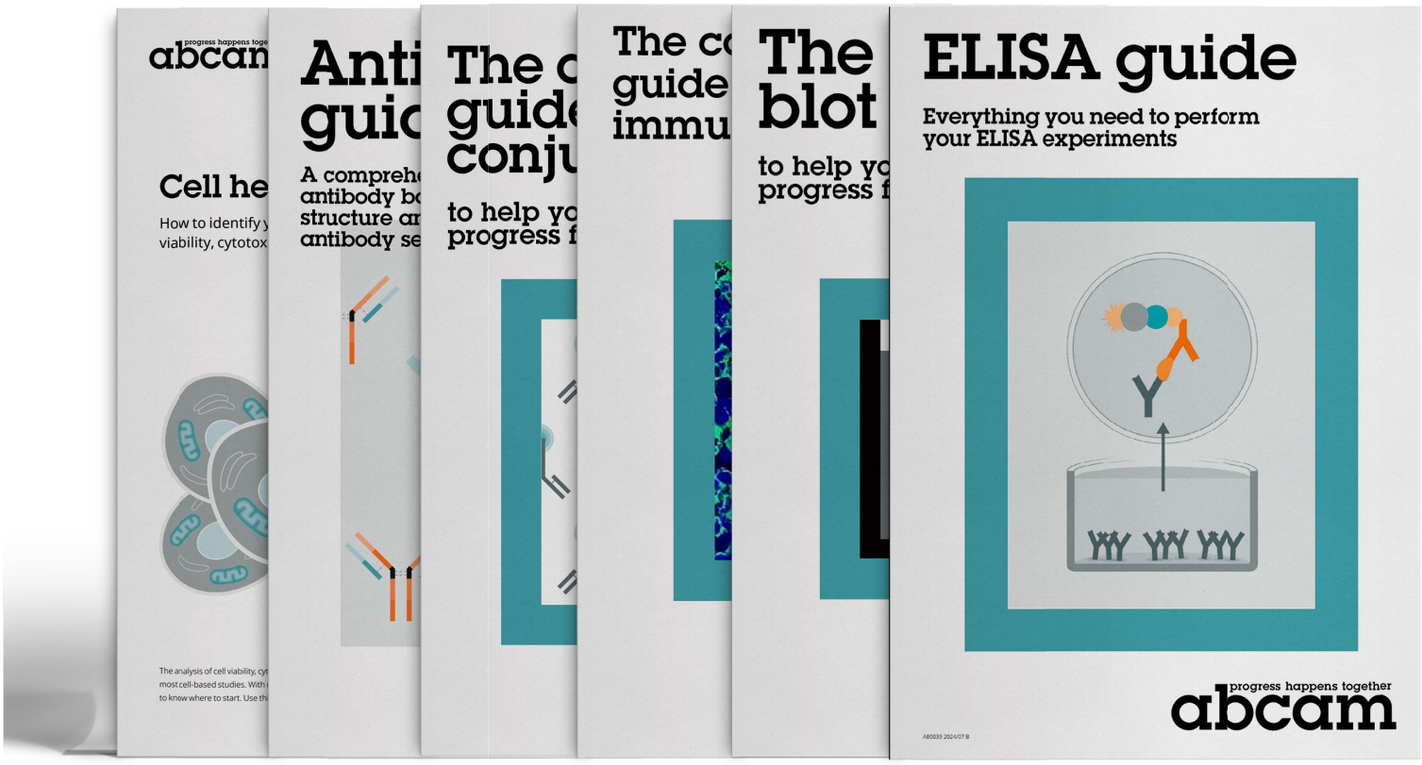
Isotype control

An isotype control can be used when working with monoclonal primary antibodies, and checks that the observed staining is not caused by non-specific interactions of the antibody with the tissue. The tissue is incubated with the antibody diluent and a non-immune antibody of the same isotype at the same concentration as the primary antibody, followed by incubation with secondary antibodies and detection reagents. Any background staining observed with this control should be negligible and distinct from specific staining.

Absorption control

An absorption control demonstrates that the antibody binds specifically to the antigen of interest by incubation with a pre-absorbed antibody instead of the primary antibody, followed by incubation with secondary antibodies and detection reagents. A pre-absorbed antibody may be produced by overnight incubation of the antibody at 4°C with a large molar excess (10-fold) of the immunogen.

Absorption controls are more reliable if the immunogen is a peptide, as antibody-protein immunogen mixtures may themselves cause high background staining in tissues due to non-specific interactions between the protein and the tissue.



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