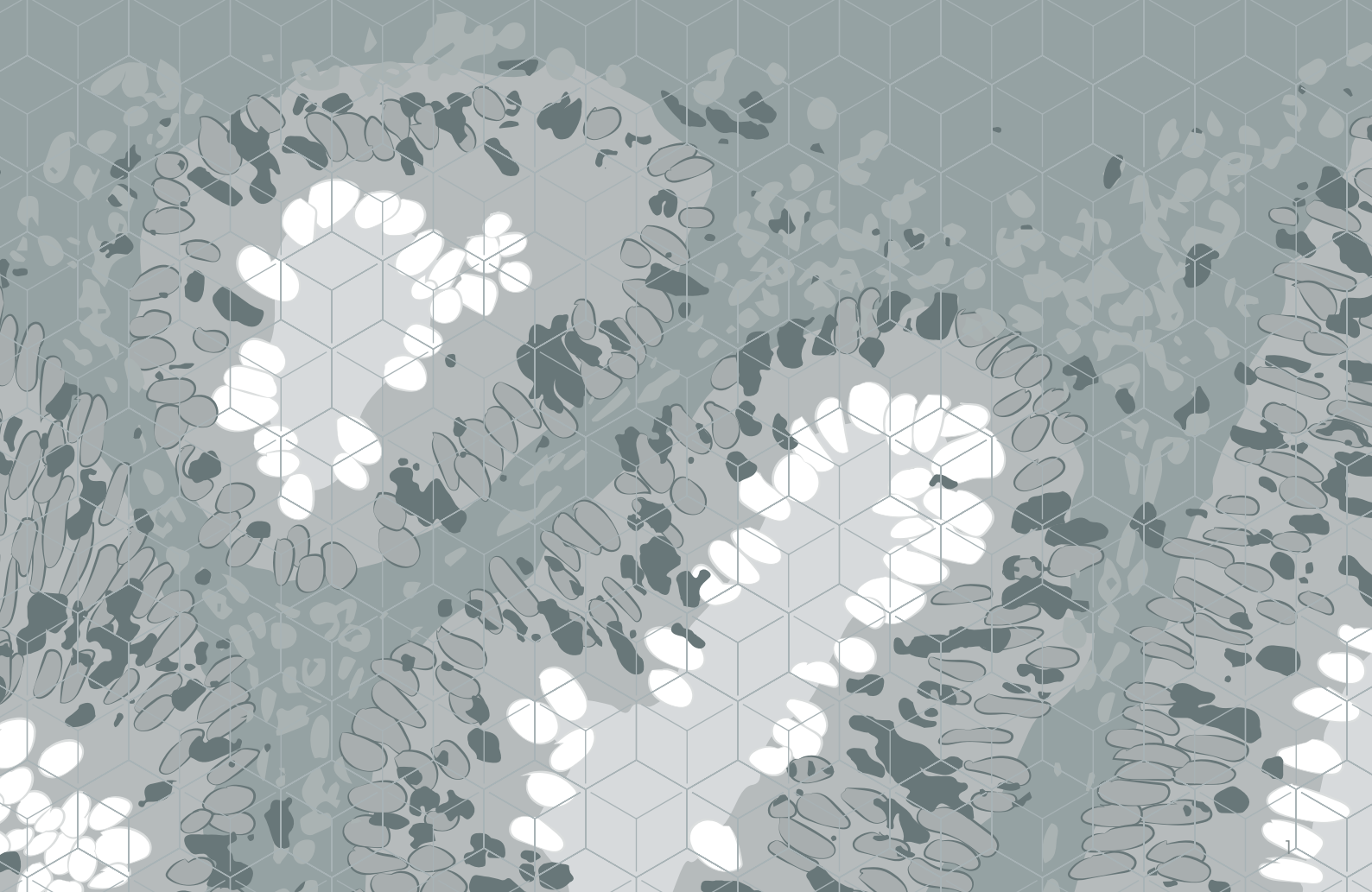
abcam

Immunohistochemistry Application Guide



Common abbreviations

ABC:	Avidin biotin complex
AEC:	3-amino-9-ethylcarbazole
AP:	Alkaline phosphatase
BCIP:	5-bromo-4-chloro-3-indolyl phosphate
BSA:	Bovine serum albumin
DAB:	3,3′ diaminobenzidine
FISH:	Fluorescence *in situ* hybridization
FITC:	Fluorescein isothiocyanate
FFPE:	Formalin-fixed, paraffin embedded
HIER:	Heat induced epitope retrieval
HRP:	Horseradish peroxidase
H_2O_2:	Hydrogen peroxide
ICC:	Immunocytochemistry
IHC:	Immunohistochemistry
IP:	Immunoprecipitation
ISH:	*In situ* hybridization
LSAB:	Labeled steptavidin biotin
KO:	Knock out
NBF:	Neutral buffered formalin
NBT:	p-nitroblue tetrazolium chloride
PBS:	Phosphate buffered saline
PFA:	Paraformaldehyde
PIER:	Proteolytic induced epitope retrieval
RabMAb:	Rabbit monoclonal antibody
TMA:	Tissue microarray
TMB:	3,3′,5,5′-tetramethylbenzidine
WB:	Western blot

Contents

Note: Products listed are for research use only

Introduction

Immunohistochemistry (IHC) is a method for detecting the location of proteins and other antigens in tissue sections using antibodies. Though less quantitative than other immunoassays such as western blotting or ELISA, IHC shows where proteins are expressed in the context of intact tissue. This is especially useful for assessing the progression and best treatment options of diseases such as cancer. In general, IHC data provide a valuable perspective that can help interpret data obtained using other methods.

The key to high quality immunohistochemical staining is the specificity of the antibody used. A highly specific antibody will bind only to the protein of interest in the tissue section. The antibody-antigen interaction is visualized using either chromogenic or fluorescent detection. In chromogenic detection, the antibody is conjugated to an enzyme that cleaves a substrate to produce a colored precipitate at the location of the protein. In fluorescent detection, the antibody is conjugated to a fluorophore that can be visualized using fluorescence microscopy.

Tips for designing a successful IHC experiment

There are a number of variables that have to be considered and optimized for every IHC experiment to ensure consistent and reproducible results. These variables are listed in Table 1.

Table 1. Immunohistochemistry variables

Variable	Factors to consider
Antigen	Species, expression level, sample type
Epitope	If the epitope has been mapped – dependence on post-translational modification
Appropriate controls	Positive and negative controls - no primary antibody, isotype control, absorption control, tissue type control
Sample preparation	Fixed or frozen
Fixation method	Perfusion or immersion (with or without freezing)
Fixative	Formaldehyde, alcohols or acetone (including concentration, pH, temperature, incubation time and diluents)
Blocking steps	Protein blocking (e.g., with serum or BSA), and, where required, blocking of biotin and/or endogenous enzymes
Antigen retrieval	Proteolytic-Induced Epitope Retrieval (PIER) or Heat-Induced Epitope Retrieval (HIER)
Detection method	Direct or indirect (with or without amplification)
Detection complex	ABC, LSAB, polymer or micro-polymer
Primary antibody	Monoclonal or polyclonal, species
Secondary antibody	Species and label
Labeling method	Fluorescent or chromogenic
Label	Fluorochromes: based on desired spectral properties Chromogens: based on detection enzyme used
Counterstain	Fluorescent: e.g., DAPI, DRAQ5™, DRAQ7™, Nuclear green, Hoechst Chromogenic: hematoxylin and eosin or histology special stain
Mounting reagent	Fluorescent: anti-fade aqueous mounting medium Chromogenic: organic/aqueous mounting medium
Visualization and analysis	Fluorescence or light microscope; analysis by eye or software-based imaging

Sample preparation

Sample preparation for an IHC experiment may include processes such as fixation, dehydration, embedding and sectioning. The two main methods of preserving tissues for IHC are paraffin embedding and freezing of the tissue. The most appropriate route of sample preparation is usually determined by one or two experimental variables. For example, if a phosphorylated epitope is being studied, tissues may need to be snap-frozen. The method of fixation often drives the design of the sample preparation workflow. Additional steps in sample preparation include antigen retrieval to unmask epitopes that have been altered by fixation, permeabilization to grant the antibody access to intracellular proteins and blocking to prevent non-specific staining.

Fixation, embedding and sectioning

Fixation prevents the autolysis and necrosis of excised tissues, preserves antigenicity, enhances the refractive index of tissue constituents and helps to preserve cellular elements during tissue processing. The fixative used is influenced by the target antigen as well as the desired detection technique (fluorescent or chromogenic). After fixation, the tissue sample is either embedded in paraffin or frozen. Embedding is important in preserving tissue morphology and giving the tissue support during sectioning (microtomy). Some epitopes may not survive harsh fixation or embedding. Some guidelines for tissue embedding are given in Table 2. During sectioning, the tissue is typically cut into thin sections (5-10 µm) or smaller pieces (for whole mount studies) to facilitate further study.

Table 2. Tissue preservation guidelines

	Paraffin-embedded tissue	Frozen tissue
Fixation	Pre-embedding	Pre/post-sectioning
Sectioning	Microtome	Cryostat
Storage	Multiple years at room temperature (Note: antigen may change over time)	1 year at -80°C (longer at -190°C)
Advantages	Preserves tissue morphology	Preserves enzyme function and antigenicity Shorter protocol (lengthy fixation step usually not required)
Limitations	Overfixation can mask the epitope	Formation of ice crystals may negatively affect tissue structure. Sections produced are often thicker than paraffin sections, increasing potential for lower resolution and poorer images
Downstream protocols	DNA and RNA for PCR amplification (extensive cross-linking prevents extraction of long nucleotide strands, free nuclei for ploidy and cell cycle analysis, cells for flow cytometry)	DNA, RNA, free nuclei for fluorescence *in situ* hybridization (FISH) or cell cycle analysis
Precautions	Duration and intensity of tissue heating should be kept to a minimum as melting temperature of paraffin wax (50-60°C) can be deleterious to staining of some antigens	Tissues should be frozen rapidly to prevent formation of ice crystals and tissues should be allowed to reach cutting temperature (-20°C) in cryostat to avoid shattering. As frozen tissues retain enzymatic activity, care should be taken to block the functions of endogenous enzymes that may affect the IHC detection method

Paraffin-embedded tissue

When generating paraffin-embedded tissue samples, the tissue must be fixed before embedding in paraffin. Fixation is achieved by perfusion or immersion immediately following dissection. The process typically takes 4 - 24 hours. Fixation for longer than 24 hours is not recommended as it may lead to overfixation, which may mask the antigen. The most suitable fixative for an IHC experiment depends on the antigen, as illustrated in Figure 1. Standardized fixatives for each type of antigen are essential for reproducible staining - an antigen that has been inappropriately fixed may not be detected. Some guidelines for the type of fixative to use are given in Table 2.

Formaldehyde

Methanol

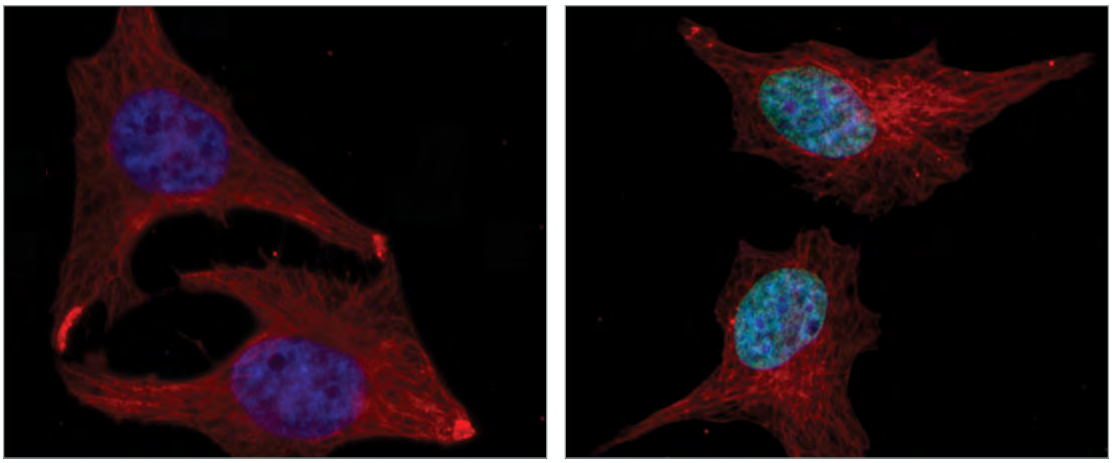


Figure 1. Effect of fixative on immunostaining patterns

Crotonylation of histone H2B K5 is clearly detected in immunocytochemistry of HeLa cells fixed with methanol (right), while no staining is observed in cells fixed in formaldehyde (left). Samples are stained with rabbit polyclonal to histone H2B (crotonyl K5) (ab177139), 1 µg/mL, and goat anti-rabbit IgG H&L (Alexa Fluor® 488) preadsorbed (ab150081), 0.5 µg/mL. Nuclei are stained with DAPI and tubulin (red) is stained with anti-alpha tubulin antibody (ab7291) and goat anti-mouse IgG H&L (Alexa Fluor® 594), preadsorbed (ab150120).

Table 3. Guidelines for choosing a fixative

Antigen	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

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. The dehydrating agent is then cleared by incubation in xylene prior to paraffin embedding. Paraffin is typically heated to 60°C and then allowed to harden overnight. Finally, the tissue is sectioned using a microtome. Tissue sections may be dried onto microscope slides and stored for extended periods at room temperature. The tissue is rehydrated before commencing the immunostaining protocol.

Frozen tissue

Frozen tissues are prepared by immersing the tissue in liquid nitrogen or isopentane, or by burying the sample in dry ice. Snap-freezing is frequently used when detecting post-translation modifications such as phosphorylation. The frozen tissue is cut using a cryostat. The sections can be stored at -80°C for up to 1 year. The frozen tissue sections may then be fixed, typically with an alcohol e.g., methanol or ethanol. As alcohols do not mask epitopes, their use avoids the need for antigen retrieval.

Antigen retrieval

Fixation can lead to protein cross-linking, which masks epitopes and can restrict antigen-antibody binding. Such masked epitopes can be retrieved (unmasked) by antigen retrieval. In the Proteolytic-Induced Epitope Retrieval (PIER) method, enzymes (such as proteinase K, trypsin or pepsin) or commercially available PIER reagents are used to restore antibody access to an epitope. The Heat-Induced Epitope Retrieval (HIER) method uses heat from a variety of sources (microwave, pressure cooker, steamer, waterbath or autoclave) to unmask epitopes. Some antigens (e.g., some cytokeratins and immunoglobulin light chains) can be retrieved more efficiently by a combination of heating and enzyme digestion.

The preferred method for optimal retrieval is dependent on the tissue, fixation and/or primary antibody and must be optimized by the histologist. A starting point for selecting the appropriate antigen retrieval method is to test two methods of HIER (for example, using citrate buffer pH 6 and Tris-EDTA pH 9) and one or two methods of PIER (for example, using proteinase K and/or trypsin). Some suggested guidelines are shown in Table 4. Conditions must be optimized for each antigen, as illustrated in Figure 2. Antigen retrieval may not be required for frozen sections.

Table 4. Epitope retrieval guidelines

	HIER	PIER
Advantages	Gentler epitope retrieval and more definable parameters	Useful for epitopes that are difficult to retrieve
pH	Citrate pH 6 buffers are often used but high pH buffers are widely applicable. Optimal pH must be determined experimentally.	Typically 7.4
Temperature	Approximately 95°C	Typically 37°C
Incubation time	20 minutes (range 10-20 minutes)	10-15 minutes (range 10-30 minutes)
Buffer composition	Depends on pH required for 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 methods such as microwaves can result in unbalanced epitope retrieval through formation of 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

	Progesterone receptor	E-cadherin
No heating		
pH 3.0 (20 mM glycine-HCl buffer)		
pH 4.5 (20 mM citrate buffer)		
pH 6.0 (20 mM citrate buffer)		
pH 7.5 (20 mM TB)		
pH 9.0 (20 mM TB)		
pH 10.5 (20 mM TB)		

Figure 2. Effects of pH on heat-induced antigen retrieval in human tissues

Adapted from Emoto K, Yamashita S and Okada Y (2005). Mechanisms of Heat-induced Antigen Retrieval: Does pH or Ionic Strength of the Solution Play a Role for Refolding Antigens? J Histochem Cytochem 53 (11):1311-21.

Buffers and antigen retrieval reagents

IHC Buffers

Product name	Size	Product code
Background reducing buffer	50 mL	ab64234
10x Citrate Buffer pH 6.0	125 mL	ab64214
100x Citrate Buffer pH 6.0	50 mL	ab64236
10x EDTA Buffer pH 8.0	125 mL	ab64216
100x EDTA Buffer pH 8.0	50 mL	ab64239
10x PBS Buffer	1 L	ab128983
25x PBS Buffer pH 7.6	125 mL	ab64026
20x PBS Buffer with Tween® 20	125 mL	ab64028
25x PBS Buffer pH 7.6	1 L	ab64246
20x PBS Buffer with Tween® 20	1 L	ab64247
25x TBS pH 7.4	125 mL	ab64203
20x TBS-T with Tween® 20	125 mL	ab64204
25x TBS pH 7.4	1 L	ab64248
20x TBS-T with Tween® 20	1 L	ab64250
10x Tris Buffer pH 10.0	125 mL	ab64222
100x Tris Buffer pH 10.0	50 mL	ab64251
Tween® 20	125 mL	ab128987

Antigen retrieval reagents / tissue pretreatments

Product name	Size	Product code
Antigen Retrieval Buffer (100X Citrate Buffer pH 6.0)	125 mL	ab93678
Antigen Retrieval Buffer (100X Citrate Buffer pH 6.0)	250 mL	ab94674
Antigen Retrieval Buffer (100X EDTA Buffer, pH 8.0)	125 mL	ab93680
Antigen Retrieval Buffer (100X EDTA Buffer, pH 8.0)	250 mL	ab94677
Antigen Retrieval Buffer (100X Tris-EDTA Buffer, pH 9.0)	125 mL	ab93684
Antigen Retrieval Buffer (100X Tris-EDTA Buffer, pH 9.0)	250 mL	ab94681
Antigen Retrieval Buffer (100X Tris Buffer, pH 10.0)	125 mL	ab93682
Antigen Retrieval Buffer (100X Tris Buffer, pH 10.0)	250 mL	ab94680
10X Tris-HCl Buffer for HIER	50 mL	ab128986
10x Heat Mediated Antigen Retrieval Solution pH 6.0	250 mL	ab973
Heat Mediated High pH Antigen Retrieving Solution	1 L	ab972
HistoReveal	15 mL	ab103720
Pepsin Solution	7 mL	ab64201
Pepsin Solution	15 mL , 125 mL	ab128991
Proteinase K	4 mL	ab64220
Trypsin (Enzymatic Pretreatment)	15 mL , 125 mL	ab128214
Trypsin Enzymatic Pretreatment Kit	7 mL	ab64205
Trypsin Enzymatic Antigen Retrieval Solution	1.6 mL concentrated liquid trypsin & 25 mL trypsin buffer	ab970

HistoReveal (ab103720) is an enzymatic antigen retrieval reagent that provides superior staining when compared to PIER using standard enzymes such as trypsin or pepsin, as shown in Figure 3. HistoReveal is specially formulated to preserve tissue morphology better than standard enzymes. As a result, less primary antibody is required.

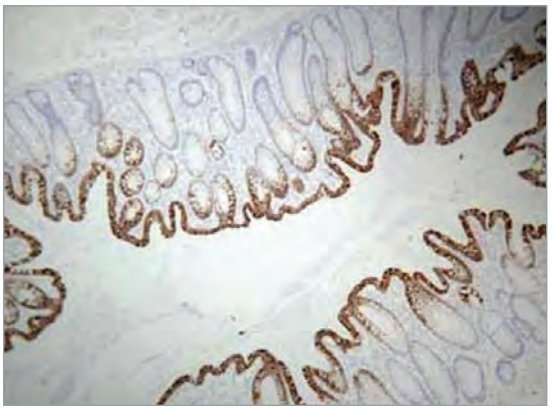


Figure 3. HistoReveal enhances staining

Cytokeratin 20 staining in colon tissue. Formalin/PFA-fixed paraffin-embedded sections following antigen retrieval using HistoReveal (ab103720).

Permeabilization

Permeabilization allows the antibody to access intracellular antigens. These include the cytoplasmic epitopes of transmembrane proteins. Permeabilization is achieved using solvents or detergents.

Solvents: Can be used after fixation with cross-linking agent e.g., paraformaldehyde. Recommended for cytoskeletal, viral and some enzyme antigens.

Detergents: These can be harsh (e.g., Triton™ X-100 or NP-40) to disrupt proteins or mild (e.g., Tween® 20, saponin or digitonin) so as not to dissolve plasma membranes. Detergent permeabilization can significantly improve antibody access to soluble nuclear antigens and antigens in the cytoplasm or on the cytoplasmic face of the plasma membrane.

Table 5. 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 - 0.2% in PBS for 10 min only
	Tween® 20, saponin, digitonin and Leucoperm	Use 0.2 - 0.5% for 10 to 30 min

Blocking

Protein blocking

Blocking with sera or a protein blocking reagent is essential to prevent non-specific binding of antibodies to tissue or Fc receptors (receptor that binds the constant region (Fc) of an antibody). Theoretically, any protein that does not have binding affinity for the target epitope can be used for blocking. In practice, some proteins are better than others because they bind more readily to non-specific sites. Serum is a common blocking agent as it contains antibodies that bind to non-specific sites. A serum matching the species of the secondary antibody is recommended. When performing multiple stains using secondary antibodies from different species, it may be necessary to use blocking sera from the species of both secondary antibodies.

Proteins such as BSA or casein may also be used to block non-specific antibody binding. These are simpler to use as there is no need to match the reagent to the species of the secondary antibody. Commercial blocking buffers, such as our 10X Blocking Buffer (ab126587), are frequently used to block non-specific antibody binding. These often have proprietary formulations that optimize performance and/or shelf life.

Sterile blocking sera

Product name	Size	Product code
Bovine Calf Serum (sterile)	25 mL	ab138477
Cat Serum (sterile)	50 mL	ab139511
Chicken Serum (sterile)	25 mL	ab138577
Donkey Serum (sterile)	50 mL	ab138579
Goat Serum (sterile)	50 mL	ab138478
Guinea Pig Serum (sterile)	50 mL	ab138480
Hamster Serum (sterile)	50 mL	ab139500
Horse Serum (sterile)	50 mL	ab139501
Mouse Serum (sterile)	50 mL	ab138705
Rabbit Serum (sterile)	50 mL	ab7487
Rat Serum (sterile)	50 mL	ab138328
Sheep Serum (sterile)	50 mL	ab138327

Non-sterile blocking sera

Product name	Size	Product code
Bovine Calf Serum	25 mL	ab7479
Cat Serum	10 mL	ab139724
Chicken Serum	25 mL	ab7477
Dog Serum	10 mL	ab139737
Donkey Serum	25 mL	ab7475
Goat Serum	50 mL	ab7481
Guinea Pig Serum	10 mL	ab7482
Hamster Serum	10 mL	ab7483
Horse Serum	25 mL	ab7484
Llama Serum	20 mL	ab139738
Mouse Serum	10 mL	ab7486
Rabbit Serum	25 mL	ab7487
Rat Serum	10 mL	ab7488
Sheep Serum	50 mL	ab7489

Biotin blocking

Blocking endogenous biotin is recommended when using an avidin/biotin-based detection system. Endogenous biotin is present in many tissues, with particularly high levels in tissues such as kidney, liver and brain.

It is blocked by pre-incubation of the tissue with avidin, followed by incubation with biotin to block additional biotin binding sites on the avidin molecule. For robust and reproducible blocking, a biotin-blocking reagent such as our Endogenous Avidin + Biotin Blocking System (ab3387) is recommended.

Protocol recommendations:

1. Block endogenous biotin prior to, or after incubation with, primary antibody but NOT after incubation with a biotinylated secondary.

2. Wet sample with blocking buffer prior to applying avidin-biotin block.

Blocking endogenous enzymes

Chromogenic detection methods usually use an enzyme conjugated to a secondary antibody to visualize antibody localization. If the enzymatic activity is also endogenous to the tissue being studied, the endogenous enzymes must be blocked before the detection step.

Peroxidase blocking

When using a horseradish peroxidase (HRP)-conjugated antibody for detection, non-specific or high background staining may occur due to endogenous peroxidase activity in tissues such as kidney, liver and those containing red blood cells (such as vascular tissue). To check for endogenous peroxidase activity, tissues can be incubated with 3,3'-diaminobenzidine (DAB) substrate prior to primary antibody incubation. If the tissues turn brown, endogenous peroxidase is present and a blocking step is required. Hydrogen peroxide (H_2O_2) is the most common peroxidase blocking agent.

Protocol recommendations:

1. Block at preferred stage of IHC protocol:
- after rehydration to water and before antigen retrieval;
- or after antigen retrieval / before primary antibody incubation;
- or after primary antibody incubation / before secondary antibody incubation;
- or after secondary antibody incubation.

   For certain antigens, like CD4 and CD8, blocking after primary and secondary incubation is recommended as H_2O_2 is detrimental to key epitopes on these proteins.

2. Incubate section in H_2O_2 (typically 0.3%) for 10-15 minutes (incubation time may be 5-60 minutes, depending on H_2O_2 concentration).

Alkaline phosphatase blocking

Endogenous alkaline phosphatase (AP) can produce high background when using an AP-conjugated antibody for detection. It can be found in kidney, intestine, osteoblasts, lymphoid tissue and placenta. Endogenous AP activity is often more prevalent in frozen tissue. 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 is observed, endogenous AP is present and blocking is necessary.

Protocol recommendations:

1. Endogenous AP can be blocked by adding levisamole with the chromogen substrate. Chromogens containing levisamole are commercially available.

2. In order to block intestinal AP, which is unaffected by levisamole, the tissue may be treated with a weak acid prior to application of the primary antibody.

Reduction of autofluorescence in IHC

When using a fluorescent label for detection, high background may be observed if the tissue is autofluorescent. Autofluorescence may be caused by the presence of fluorescent compounds in the tissue, such as flavins and porphyrins. These compounds may be extracted 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. The fixation step may also induce autofluorescence. This often occurs when using aldehyde fixatives, which react with amines to generate fluorescent products. Tests should be carried out to ensure that the tissue being studied is not inherently fluorescent or that fixation steps do not induce autofluorescence. Potential methods to reduce autofluorescence are outlined in Table 6.

Table 6. Autofluorescence blocking guidelines

Method 1	Use frozen tissue sections to reduce possibility of induced autofluorescence during fixation
Method 2	Use fixatives that do not contain aldehydes, such as Carnoy's solution (if generating paraffin sections)
Method 3	Block aldehydes during fixation by treating tissue with sodium borohydride or glycine/lysine
Method 4	Treat tissue with quenching dyes such as pontamine sky blue, Sudan black, trypan blue or FITC block

If endogenous autofluorescence cannot be blocked, a chromogenic detection system may be preferable.

Blocking cross-reactive antigens

When staining mouse tissues with mouse primary antibodies, high background is often observed as endogenous mouse IgG and Fc receptors are detected by the secondary antibody targeting the (exogenous) mouse primary antibody. This background binding can be reduced using F(ab) fragments. (*Note*: a F(ab) fragment is a single variable domain of an antibody that binds to an antigen but has no Fc region.) For robust and reproducible staining, a Mouse on Mouse Polymer IHC kit such as ab127055 is recommended, as shown in Figures 4 and 5.

Key benefits of our Mouse on Mouse Polymer IHC kit (ab127055) are:
- Minimal background
- Strong staining through polymer-based detection
- Biotin-free detection in biotin-enriched tissue
- Simple and reliable protocol for reproducible experiments

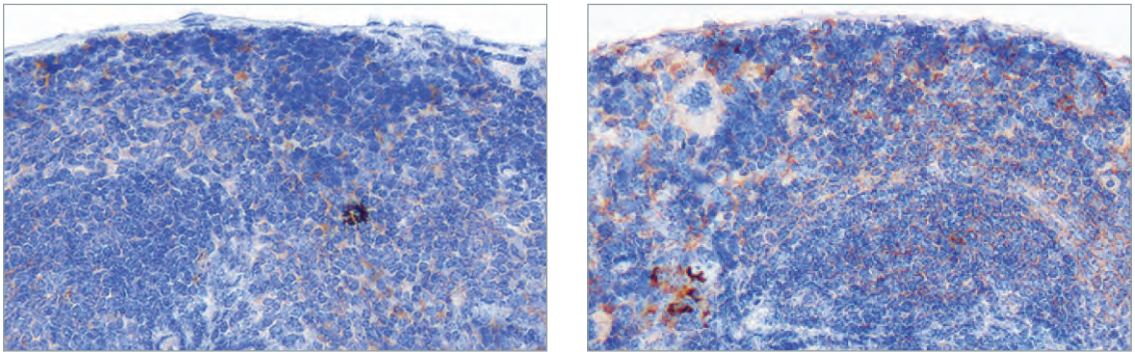


Figure 4. Reduced endogenous mouse IgG background using ab127055

Negative control images using Mouse on Mouse Polymer IHC kit (ab127055) (left) and EXPOSE detection kit (ab80436), which is not suitable for use on mouse tissues (right). ab127055 reduces endogenous mouse IgG background staining. Both images show mouse spleen formalin/PFA-fixed paraffin-embedded tissues.

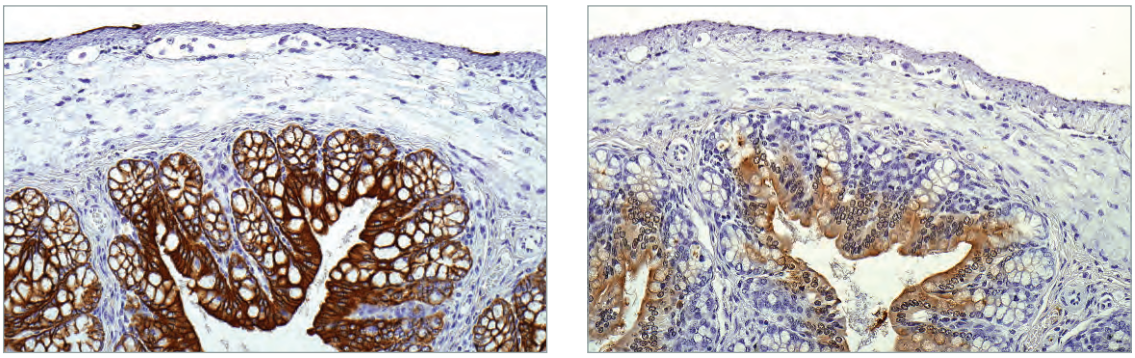


Figure 5. Stronger staining with Mouse on Mouse detection using ab127055

Mouse Pan CK (Clone AE1/AE3) antibody staining using Mouse on Mouse Polymer IHC kit (ab127055) (left) compared with ABC Mouse on Mouse system (ab80436). (right) ab127055 gives stronger staining. Both images show mouse colon formalin/PFA-fixed paraffin-embedded tissues.

Reagents for blocking

Product name	Size	Product code
Endogenous Avidin/Biotin Blocking Kit	15 mL	ab64212
Endogenous Avidin + Biotin Blocking System	15 mL avidin, 15 mL biotin	ab3387
FITC Protein Blocking Agent (PBA)	6 mL	ab128980
Hydrogen Peroxide Blocking Reagent	125 mL	ab64218
Hydrogen Peroxide Blocking Reagent	60 mL	ab94666
Protein Block	125 mL	ab64226
Protein Block	60 mL	ab156024
Sea Block Buffer	500 mL	ab166951
Aqua Block	500 mL	ab166952

Immunostaining

Immunostaining (or immunodetection) relies on the specificity of the primary antibody for the target antigen. The antibody is detected either directly, through a label that is directly conjugated to the primary antibody, or indirectly, using a labelled secondary antibody that has been raised against the host species and antibody type and subtype of the primary antibody. The antibody is visualized using either a fluorescent label or an enzyme that converts a soluble substrate into an insoluble chromogenic product. An outline of immunostaining is show in Figure 6. Direct immunostaining methods do not require a secondary antibody incubation. In fluorescent detection, the addition of substrate is omitted.

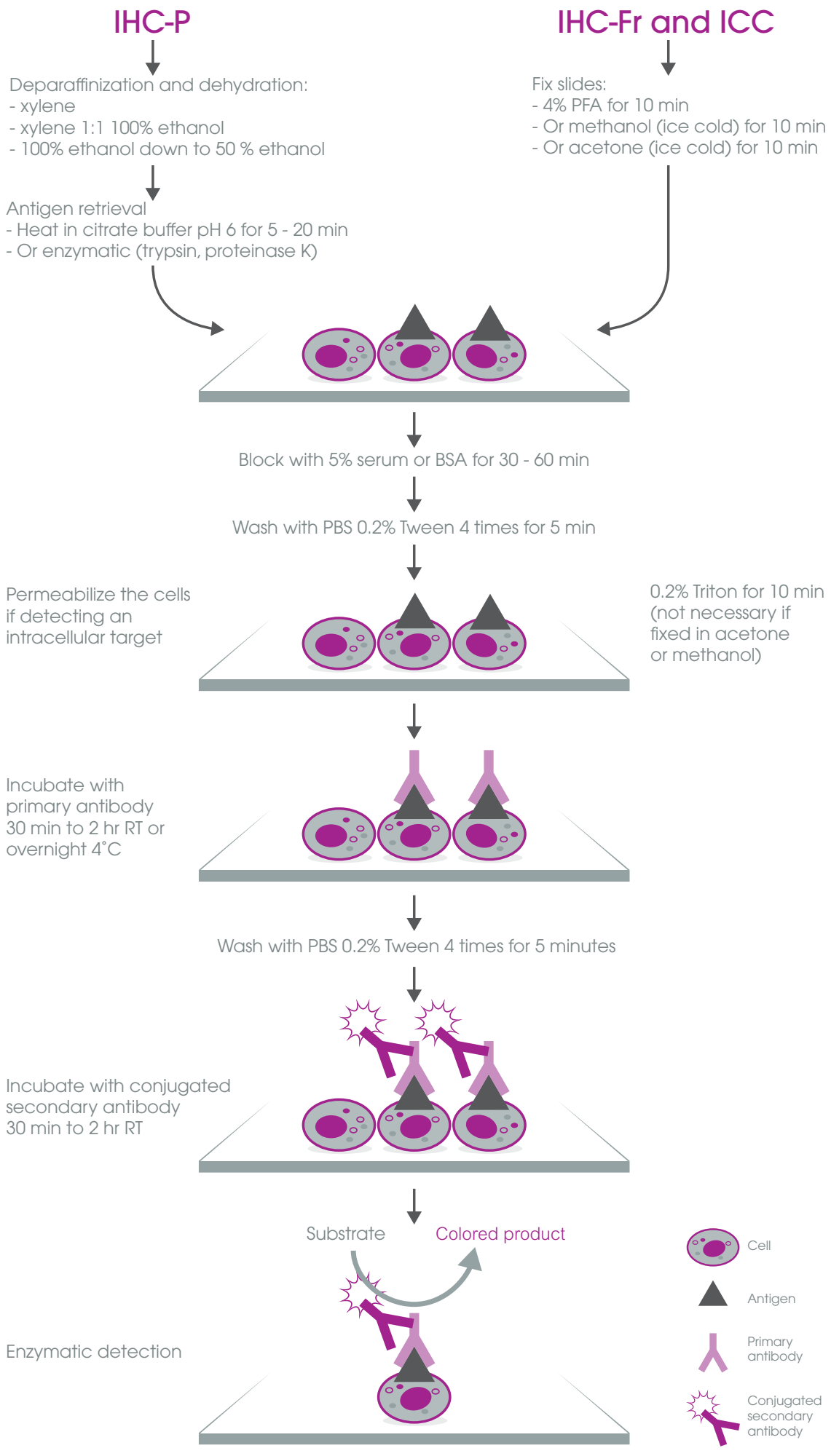


Figure 6. A typical immunostaining protocol

Primary antibody selection and optimization

The most critical decision when designing an IHC experiment is the selection of the primary antibody. The key factors that must be considered are discussed below. Once the primary antibody has been chosen, optimization is essential.

Antibody specificity

Specificity of the primary antibody for the epitope of interest is usually determined experimentally. 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 (1) recognition of a single band in western blotting, (2) staining patterns that are consistent with known localization of the protein of interest in control cells or tissues and (3) lack of staining in tissues or cells known not to express the protein. Comparison of the immunogen sequence to other proteins using alignment tools such as BLAST may give some indication about antibody specificity but is not conclusive. Ideally, the antibody should recognize the target antigen in the species of interest. If this information is unavailable, sequence comparison of the immunogen with the corresponding region in the protein from the species of interest may give some indication of specificity.

Proof of use in IHC

An antibody that recognizes its target protein in western blotting experiments, which are run under denaturing conditions, may not always recognize the antigen in IHC, where the antigen is more likely to be in its native (3D) form. An antibody that has been shown to work in IHC or immunocytochemistry (ICC) is preferable. It is important to note that fixation and antigen retrieval methods also have significant impact on the ability of an antibody to recognize the epitope of interest in an IHC experiment.

Clonality

As monoclonal antibodies are produced from a single B cell clone, they represent a homogeneous population that binds to a single epitope. As a result, they are less likely to cross-react with other proteins (provided that the clone recognizes a unique epitope) and therefore produce less background staining than polyclonal antibodies. The production of monoclonals from a hybridoma also means that there is less variation between different antibody lots than with polyclonal antibodies.

Polyclonal antibodies are heterogeneous populations that can recognize multiple epitopes. They are therefore more tolerant of changes in protein conformation (resulting, for example, from fixation or changes in temperature). They are also more stable over a range of pH and salt concentrations than monoclonal antibodies.

Host species

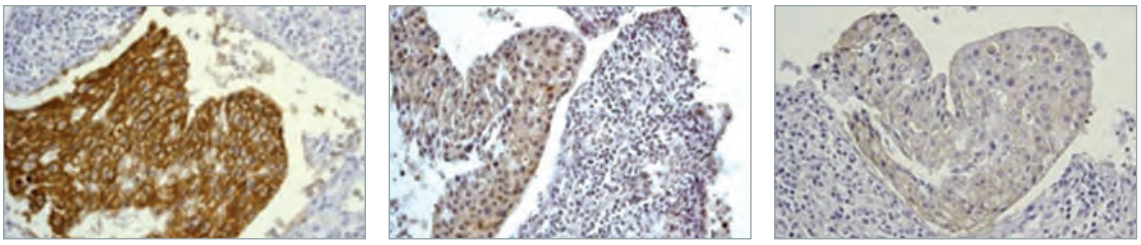
Ideally, the primary antibody should be raised in a host species that is different to the species of the sample in order to avoid cross-reactivity with endogenous immunoglobulins in the tissue.

Antibody optimization

The quality of staining is influenced by the primary antibody concentration, the diluent used, the incubation time and temperature. All of these variables may need to be optimized for each antibody and sample in order to achieve specific staining with minimal background. Typically, antibody concentration is varied while maintaining a constant incubation time and temperature. Longer incubation times may be used to ensure that the antibody penetrates the tissue. Longer incubation times can be combined with lower temperatures to promote specific binding.

RabMAb® antibody advantages for IHC

We offer high-quality rabbit monoclonal antibodies developed using our patented RabMAb technology. As monoclonals, RabMAb® primary antibodies detect a single epitope and are therefore less likely to cross-react with other proteins. At the same time, we have observed that RabMAb® primary antibodies bind to their target with greater affinity, enabling higher signal-to-noise ratio than mouse monoclonal antibodies at a given antibody concentration. RabMAb® primary antibodies typically provide more specific and sensitive detection of their target protein with low background, making them ideal for demanding applications like IHC on FFPE tissues (Figure 7). RabMAb® antibodies typically allow higher working dilutions (5 - 10X on average) and can be used with various tissue fixatives such as FFPE at a minimal level of pretreatment. Additionally, when used along with a mouse monoclonal, dual staining with two monoclonal antibodies can be performed for high quality double staining on the same tissue sample.



HER2/ ErbB2 RabMAb® antibody 3 ng/mL

Rabbit polyclonal antibody 20 ng/mL (vendor A)

Mouse monoclonal antibody 30 ng/mL (vendor B)

Figure 7. HER2 RabMAb® primary IHC antibody comparison

A comparison of our HER2/ ErbB2 RabMAb® antibody against leading commercially available HER2/ErbB2 rabbit polyclonal (Vendor A) and mouse monoclonal (Vendor B) on FFPE human breast carcinoma tissue. Recommended IHC protocol and dilution factor were used for each case. The antibody concentration used is shown next to the image for each stain.

Knockout (KO) validated antibodies

With the increasing need for highly specific antibodies, we are introducing KO validation as a standard quality control test on our antibodies. Antibody specificity is confirmed by testing the antibody of interest in a knockout cell line (which does not express the protein). This data is compared side-by-side against a normal (wild type) cell line. If the antibody is truly specific, the antibody should show no staining in the knockout cell line and a specific signal in the normal cell line (Figure 8). Knock out validations offer a true negative control to confirm the antibody specificity to the protein of interest. When you purchase one of our KO validated antibodies, you can trust that the antibody has been validated in the recommended applications and species, and that its specificity has also been confirmed through our in-house knockout validation approach.

Find out more at www.abcam.com/KO

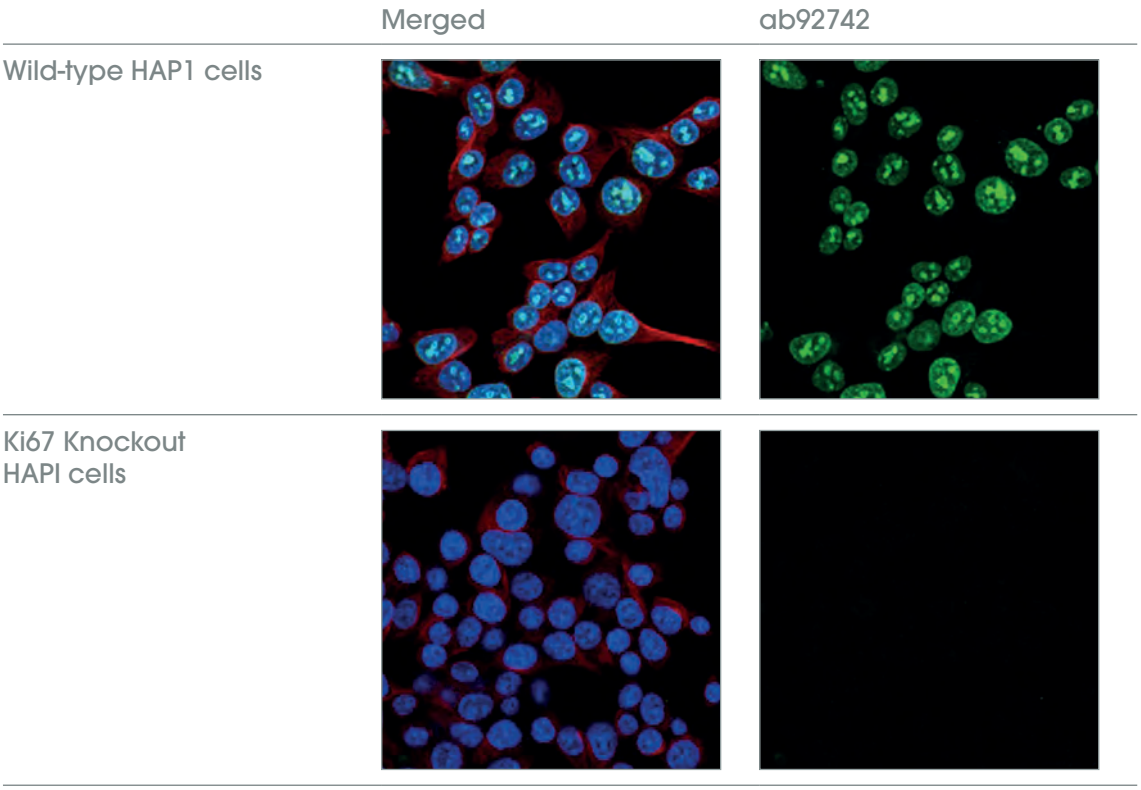


Figure 8. Knockout specificity testing by immunohistochemistry for Ki67 antibody (ab92742)

ab92742 specifically recognizes Ki67 in wild-type HAP1 cells (top right panel) and not in Ki67 knockout HAP1 cells (bottom left panel). The cells were fixed with 100% methanol (5 min), permeabilized with 0.1% Triton™ X-100 for 5 mins and blocked with 1% BSA/10% normal goat serum/0.3 M glycine in 0.1% PBS-Tween for 1 hr. Cells were incubated with ab92742 at 1µg/mL and anti-alpha tubulin antibody (Alexa Fluor® 594) (ab195889) at 1:250 dilution (shown in pseudo color red) overnight at +4°C, followed by a further incubation at room temperature for 1 hr with a goat secondary antibody to rabbit IgG (Alexa Fluor® 488) (ab150081) at 2 µg/mL (shown in green). Nuclear DNA was labelled in blue with DAPI.

Direct vs indirect detection

Once a primary antibody is bound to the antigen of interest, it can be detected either directly or indirectly. For direct detection methods, the primary antibody is directly conjugated to a label. For indirect detection, the primary antibody is detected by a labeled secondary antibody. Indirect methods may include amplification steps to increase signal intensity.

Direct detection is suitable for studying highly expressed antigens. For direct detection, the primary antibody can be conjugated to an enzyme, such as HRP or AP, or a fluorochrome. The benefit of direct detection is that an additional incubation step with a secondary reagent is unnecessary. Another significant benefit of direct detection is increased flexibility in the design of multicolor experiments. Our rapid one-step antibody conjugation kits allow you to create your own directly-labeled primary antibodies. Choose from enzymatic, fluorescent, biotin or gold labels.

For additional information, visit www.abcam.com/conjugation

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. However, the use of a secondary antibody requires additional blocking steps and controls. The signal may be amplified further by using avidin or streptavidin with biotinylated secondary antibodies (and is discussed in the following sections).

Secondary antibodies for IHC

During indirect detection, the secondary antibody should be directed against the species in which the primary antibody was raised. For example, if using a primary antibody raised in rabbit, an anti-rabbit secondary antibody raised in a species other than rabbit must be used. It is also important that the secondary antibody has been raised against the isotype of the primary antibody. Affinity-purified antibodies are the most popular as they provide the lowest amount of non-specific binding. However, IgG fractions sometimes contain very high affinity antibodies and may be useful when an antigen is in low abundance.

The use of pre-adsorbed secondary antibodies can reduce non-specific background as they are less likely to show species cross-reactivity or to react with endogenous antigens of the species against which they have been pre-adsorbed. The secondary antibody should be pre-adsorbed against the species in which the sample originated. For example, a secondary antibody pre-adsorbed against human serum should be used when staining human tissues or cell lines.

For more information, please go to www.abcam.com/pre-adsorbed

$F(ab')_2$ fragment secondary antibodies are recommended for staining of tissues rich in Fc receptors (e.g., spleen, thymus, blood). (*Note*: $F(ab')_2$ fragments have 2 antigen binding domans linked by disulfide bonds, but no Fc region.) As these secondary antibodies are smaller and therefore penetrate tissues more easily, they are particularly useful for multiple IHC staining.

Secondary antibodies can be labeled with enzymes (HRP, AP), fluorochromes or biotin. We offer a range of biotinylated secondary antibodies for use in ABC (avidin-biotin complex) detection systems.

Optimized IHC secondary antibodies

Product name	Size	Product code
Biotinylated Goat anti Mouse IgG (H+L) (Ready-To-Use)	125 mL	ab64255
Biotinylated Goat anti Rabbit IgG (H+L) (Ready-To-Use)	125 mL	ab64256
Biotinylated Goat anti Mouse & Rabbit IgG (H+L)	125 mL (Ready-To-Use)	ab64257
Biotinylated Goat anti Mouse & Rabbit IgG (H+L)	60 mL (Ready-To-Use)	ab128977
Mouse polyclonal to Peroxidase anti-Peroxidase complex/PAP antibody	100 µL	ab21867

Discover other secondary antibodies at www.abcam.com/secondaries

Chromogenic detection

Chromogenic detection uses enzymes that convert soluble substrates into insoluble, chromogenic products. These enzymes are usually attached to secondary antibodies but can also be attached to primary antibodies for direct detection. The most commonly used enzymes are HRP, which converts DAB into a brown product, and AP, which converts 3-amino-9-ethylcarbazole (AEC) into a red product. Chromogenic detection is usually more sensitive than fluorescent detection. Furthermore, unlike fluorophores, the colored precipitates are photostable, allowing storage of the slides for many years. Unlike fluorescent detection, which 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. Four main methods of indirect chromogenic detection are widely used today.

Avidin-Biotin Complex (ABC)

The ABC method (Figure 9) relies on biotinylated secondary antibodies that act as links between a tissue-bound primary antibody and an avidin-biotin-reporter enzyme complex. As avidin is tetravalent, it forms large complexes containing multiple copies of the biotinylated reporter enzyme, resulting in high signal intensity.

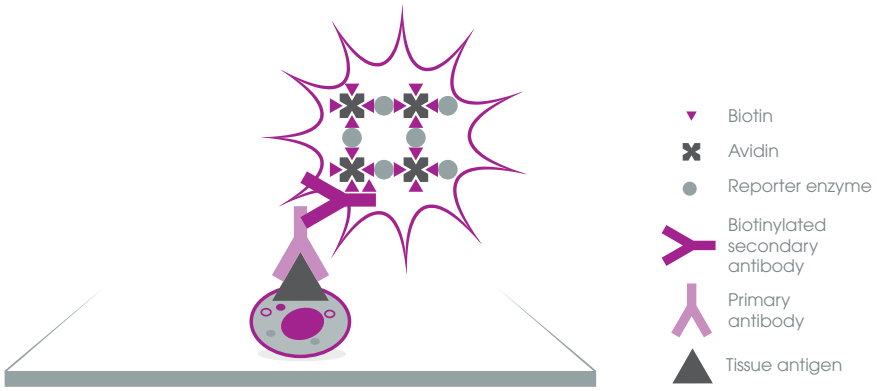


Figure 9. Avidin-Biotin Complex (ABC) method

Labeled Steptavidin-Biotin (LSAB)

The LSAB method (Figure 10) is similar to the ABC method because it uses a biotinylated secondary antibody that links a primary antibody to a streptavidin-enzyme conjugate. While streptavidin is also tetravalent, it is not glycosylated and has a more neutral isoelectric point than avidin, resulting in less non-specific tissue binding.

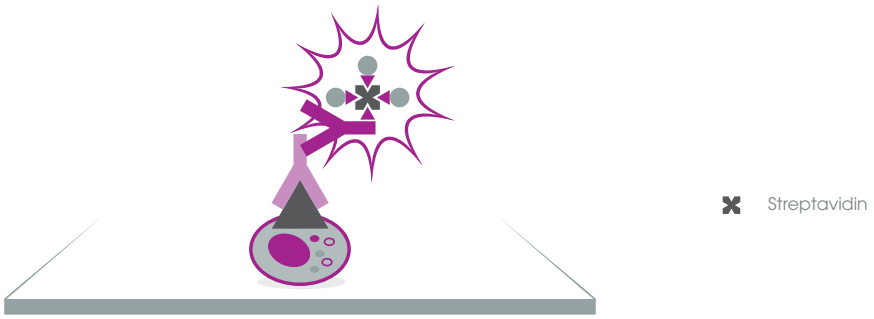


Figure 10. Labeled Streptavidin-Biotin (LSAB) method

We offer a number of kits that use the LSAB detection system. Choose our HRP Plus detection kit for enhanced sensitivity (ab93697).

Mouse and rabbit specific kits (anti-polyvalent)

Product name	Size	Product code
Mouse and Rabbit specific HRP/DAB (ABC) Detection IHC Kit	15 mL	ab64264
Mouse and Rabbit specific HRP/DAB Plus (ABC) Detection IHC Kit	60 mL, 125 mL	ab93697
Mouse and Rabbit specific HRP/AEC (ABC) Detection IHC Kit	15 mL	ab93705
Mouse and Rabbit specific HRP (ABC) Detection IHC Kit	125 mL, 1 L	ab93677
Mouse and Rabbit specific AP (ABC) Detection IHC Kit	60 mL, 125 mL	ab93695
Mouse and Rabbit specific AP/Fast Red (ABC) Detection IHC Kit	15 mL	ab128967

Rabbit specific kits

Product name	Size	Product code
Rabbit specific HRP/DAB (ABC) Detection IHC Kit	15 mL	ab64261
Rabbit specific HRP/AEC (ABC) Detection IHC Kit	15 mL	ab64260
Rabbit specific AP (ABC) Detection IHC Kit	60 mL, 125 mL	ab128972

Mouse specific kits

Product name	Size	Product code
Mouse specific HRP/DAB (ABC) Detection IHC Kit	15 mL	ab64259
Mouse specific HRP/AEC (ABC) Detection IHC Kit	15 mL	ab64258

Note: Our ABC detection IHC kits use an LSAB detection system.

Polymer

Although biotin-based detection systems are still widely used, there are a number of limitations associated with these methods. The key challenge is that the presence of endogenous biotin can lead to significant background staining in certain tissues (e.g., kidney or brain). Furthermore, while formalin fixation and paraffin embedding reduce the levels of endogenous biotin, procedures such as HIER can result in the recovery of endogenous biotin as an undesirable side effect. Background staining from biotin is a significant problem when staining frozen sections, where levels of endogenous biotin tend to be higher than in paraffin-embedded specimens. Methods to block endogenous biotin are often partially effective and add another level of complexity to the IHC procedure. Non-biotin based detection methods were introduced to offer a solution to the challenge of endogenous biotin background. These methods also have simpler protocols and generate comparable, if not better, staining to ABC methods. Polymer-based methods use a dextran backbone to which multiple enzyme molecules and secondary antibodies are attached. The dextran backbone-secondary antibody complex then binds to the primary antibody (Figure 11).

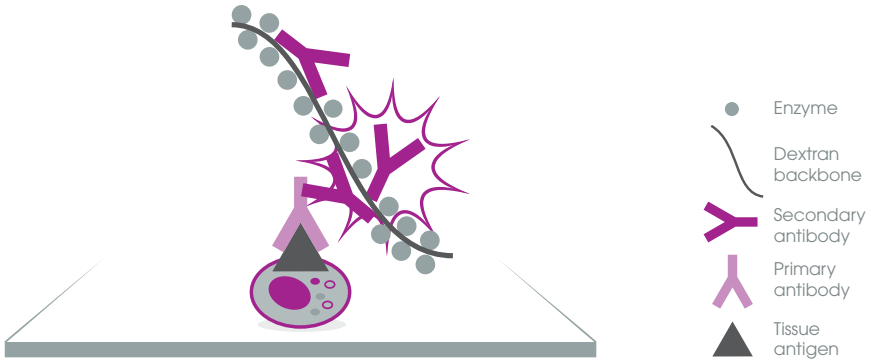


Figure 11. Polymer method

Micropolymer

More recently, micropolymer (or compact polymer)-based detection methods have been developed (Figure 12). The enzyme is polymerized directly onto the secondary antibody, forming a smaller detection complex. Unlike conventional detection polymers, micropolymers do not have a tendency to aggregate as they do not contain a dextran backbone. The main advantages offered by the smaller micropolymers are greater sensitivity through better tissue penetration than the polymer methods and improved signal-to-noise ratio with no background staining from endogenous biotin.

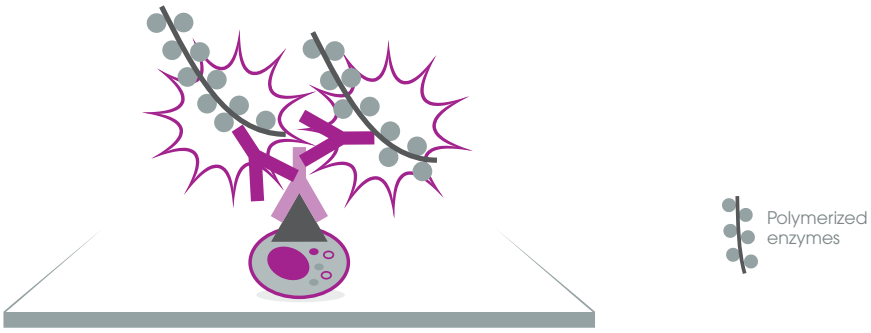


Figure 12. Micro-polymer method

Discover the advantages of using a micro-polymer/compact-polymer detection system. Our EXPOSE range offers greater sensitivity as well as improved signal-to-noise ratio.

Product name	Size	Product code
EXPOSE Mouse and Rabbit specific HRP/DAB detection IHC kit	15 mL, 60 mL, 125 mL	ab80436
EXPOSE Mouse and Rabbit specific HRP/AEC detection IHC kit	15 mL, 60 mL, 125 mL	ab93686
EXPOSE Mouse and Rabbit specific AP (red) detection IHC kit	15 mL, 60 mL, 125 mL	ab94734
EXPOSE Mouse and Rabbit specific HRP/DAB or AEC detection IHC kit	1 L	ab93702
EXPOSE Rabbit specific HRP/DAB detection IHC kit	15 mL, 60 mL, 125 mL	ab80437
EXPOSE Rabbit specific HRP/AEC detection IHC kit	15 mL, 60 mL, 125 mL	ab94361
EXPOSE Rabbit specific AP (red) detection IHC kit	15 mL, 60 mL, 125 mL	ab94737
EXPOSE Mouse specific AP (red) detection IHC kit	15 mL, 60 mL, 125 mL	ab94740

Chromogenic multicolor detection systems

Detection of multiple antigens using chromogenic methods usually involves long and complicated protocols. Our muticolor enzymatic IHC kits have been designed for optimal staining of two or three antigens on mammalian tissue sections using an optimized protocol that avoids multiple sequential staining steps. Other benefits of our multicolor enzymatic IHC kits are:

- Biotin blocking not required: polymer-based system
- Better signal-to-noise ratio: clear visualization of staining and morphology
- Choice of color combinations: red, green, purple, brown and black
- Use of rodent antibodies on rodent tissue: protocols for blocking endogenous mouse or rat IgG

Kit name and selection key

Kit primary antibody key:
- G: Goat, M: Mouse, R: Rabbit, Rt: Rat

Kit chromogen name and color key:
- DAB: Brown
- AP/Red & Fast-Red: Red
- HRP/Green: Green
- DAB/Ni: Black
- BCIP: Blue
- AEC: Red

Size guidelines if using 100 µL polymer conjugate per slide:
- DoubleStain kits: 12 mL (~120 slides), 36 mL (~360 slides), 120 mL (~1200 slides)
- TripleStain kits: 24 mL (~120 slides), 72 mL (~360 slides), 240 mL (~1200 slides)

Double-staining kits for human tissue

Product name	Size	Product code
DoubleStain IHC kit: G&M on human tissue (DAB & AP/Red)	12 mL, 36 mL, 120 mL	ab183159
DoubleStain IHC kit: G&M on human tissue (BCIP & AEC)	12 mL, 36 mL, 120 mL	ab183271
DoubleStain IHC kit: G&M on human tissue (HRP/Green & AP/Red)	12 mL, 36 mL, 120 mL	ab183272

Double-staining kits for animal tissue

Product name	Size	Product code
DoubleStain IHC kit: M&M on rodent tissue (DAB & AP/Red)	12 mL, 36 mL, 120 mL	ab183273
DoubleStain IHC kit: M&M on rodent tissue (BCIP & AEC)	12 mL, 36 mL, 120 mL	ab183274
DoubleStain CL IHC kit: M&M on rodent tissue (HRP/Green & AP/Red)*	12 mL, 36 mL, 120 mL	ab183275
DoubleStain CL IHC kit: M&M on rodent tissue (DAB & FastRed)*	12 mL, 36 mL, 120 mL	ab183276
DoubleStain IHC kit: M&Rt on mouse tissue (DAB & AP/Red)	12 mL, 36 mL, 120 mL	ab183277
DoubleStain IHC kit: M&Rt on mouse tissue (BCIP & AEC)	12 mL, 36 mL, 120 mL	ab183278
DoubleStain IHC kit: M&Rt on mouse tissue (HRP/Green & AP/Red)	12 mL, 36 mL, 120 mL	ab183279

* Note: CL kits are designed for ease of use in co-localization experiments

Double-staining kits for human and rodent tissue

Product name	Size	Product code
DoubleStain IHC kit: G&Rt on human/ mouse tissue (DAB & AP/Red)	12 mL, 36 mL, 120 mL	ab183280
DoubleStain IHC kit: G&Rt on human/ mouse tissue (BCIP & AEC)	12 mL, 36 mL, 120 mL	ab183281
DoubleStain CL IHC kit: G&Rt on human/mouse tissue (HRP/Green & AP/Red)*	12 mL, 36 mL, 120 mL	ab183282
DoubleStain IHC kit: R&Rt on human/ mouse tissue (DAB & AP/Red)	12 mL, 36 mL, 120 mL	ab183283
DoubleStain IHC kit: R&Rt on human/ mouse tissue (BCIP & AEC)	12 mL, 36 mL, 120 mL	ab183284
DoubleStain IHC kit: R&Rt on human/ mouse tissue (HRP/Green & AP/Red)	12 mL, 36 mL, 120 mL	ab183285

* Note: CL kits are designed for ease of use in co-localization experiments

Triple-staining kits for human tissue

Product name	Size	Product code
TripleStain IHC kit: M&M&R on human tissue (DAB, AP/Red & HRP/Green)	24 mL, 72 mL, 240 mL	ab183286
TripleStain IHC kit: M&M&R on human tissue (DAB, AP/Red & DAB/Ni)	24 mL, 72 mL, 240 mL	ab183287
TripleStain IHC kit: R&R&M on human tissue (DAB, AP/Red & HRP/Green)	24 mL, 72 mL, 240 mL	ab183288
TripleStain IHC kit: R&R&M on human tissue (DAB, AP/Red & DAB/Ni)	24 mL, 72 mL, 240 mL	ab183289
TripleStain IHC kit: M&R&G on human tissue (DAB, AP/Red & HRP/Green)	24 mL, 72 mL, 240 mL	ab183290
TripleStain IHC kit: M&R&G on human tissue (DAB, AP/Red & DAB/Ni)	24 mL, 72 mL, 240 mL	ab183291

Enzymes and and chromogens

Additional factors for consideration in chromogenic detection are the choice of enzyme and chromogenic substrate. A number of different chromogens are available for each detection enzyme. Table 7 summarizes the key features of commonly used enzymes and chromogens/substrates.

Table 7. Enzymes and substrates / chromogens for IHC

Enzyme	Chromogen / substrate	Color	Mounting media	Advantages	Disadvantages
HRP	AEC	Red	Aqueous	Intense color; contrasts well with blue in double staining	Endogenous peroxidase activity in tissue can lead to false positive staining
	DAB	Brown	Organic	Intense color; permanent	
	DAB + nickel enhancer	Black	Organic	Intense color; permanent	
	TMB	Blue	Aqueous	Intense color; permanent	
AP	BCIP/NBT	Blue	Organic	Intense color	Endogenous AP activity in tissue can lead to false positives Fast-Red and Fast Blue TR prone to fading
	Naphthol AS-MX phosphate + Fast Blue BB	Blue	Aqueous	Less intense, good for double staining	
	Naphthol AS-MX phosphate + Fast-Red TR	Red	Aqueous	Less intense, good for double staining	
	Naphthol AS-MX phosphate + new fuchsin	Red	Organic	Intense color	
Glucose oxidase	NBT	Blue	Organic	No endogenous enzyme activity	Low staining intensity High antibody concentrations needed

Substrate and chromogen products

Product name	Size	Product code
AEC Single/Plus	30 mL	ab103742
AEC Substrate System (Ready to Use)	125 mL	ab64252
Alkaline Phosphatase chromogen (BCIP/TNBT)	100 mL	ab7413
Alkaline Phosphatase chromogen (BCIP/NBT)	100 mL	ab7468
Alkaline Phosphatase Enhancer	250 mL	ab671
DAB Enhancer	10 mL	ab675
DAB Substrate Kit	125 mL	ab64238
DAB Substrate Kit	60 mL	ab94665
Fast-Red Substrate System	60 mL	ab128979
Permanent Fast-Red Substrate System	60 mL, 125 mL	ab128992
Liquid Fast-Red Substrate Kit	60 mL	ab128981
Liquid Fast-Red Substrate Kit	125 mL	ab64254
StayGreen/AP (Alcohol and xylene substitute compatible)	30 mL	ab156428
StayRed/AP (Alcohol and xylene compatible)	30 mL	ab103741
Steady DAB/Plus	200 mL	ab103723
Streptavidin Alkaline Phosphatase (Ready to Use)	125 mL	ab64268
Streptavidin Peroxidase (Ready to Use)	125 mL	ab64269

Fluorescent detection

Fluorescent detection (immunofluorescence) methods use fluorochrome labels, which emit light of a longer wavelength when excited by light of a specific wavelength. Fluorescent detection is commonly used to visualize multiple antigens simultaneously. The fluorochrome may be conjugated directly to the primary or secondary antibody or to streptavidin.

When designing multicolor experiments, two key parameters must be considered. Firstly, spectral overlap between the fluorochromes should be limited as much as possible. Secondly, if indirect detection methods are employed, cross-reactivity between the detection reagents should be avoided. 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. In this method, the tissue is incubated with the non-biotinylated antibody first, followed by incubation with the corresponding flurochrome-conjugated secondary antibody. The tissue is then incubated with the biotinylated antibody, followed by incubation with a streptavidin-conjugated fluorophore, which binds to the biotin conjugated to the primary antibody. This method is susceptible to high background staining from endogenous biotin, particularly when using frozen tissues.

Counterstaining

After immunostaining the antigen in a tissue, additional stains are often used to counterstain specific cellular and tissue morphologies or structures in order to aid localization of the primary label. For example, counterstaining with an iron stain improved the interpretation of caspase 3 and CD68 antibody staining in human spleen tissue in human macrophages in tissues containing excess iron (Figure 13). Special stains are also useful for the evaluation of disease states.

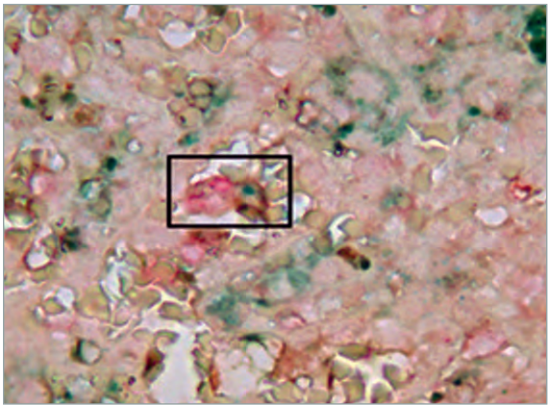


Figure 13. Iron Stain Kit (Prussian Blue)

Iron Stain Kit (ab150674) with its blue reaction product used in combination with directly conjugated caspase 3 antibody with DAB (brown) and CD68 antibody with AP red (red/pink) on FFPE human spleen tissue (63x). Used to investigate aspects of apoptosis in human macrophages of different tissues containing excess iron. Washington S, Johnson PY, Beauchamp MD, Handa P, and Mzumara A (2014). Multicolor Enzymatic IHC Assays for FFPE Tissue. BioTechniques 56, 334–336.

Common counterstains and their targets

Type	Dye	Target	Color	Product code
Chromogenic	Mayers Hematoxylin	Nuclei	Blue to violet	ab128990
Chromogenic	Nuclear fast red (Kernechtrot)	Nucleic acids	Red	-
Chromogenic	Methyl green	Nucleic acids	Green	-
Fluorescent	DRAQ5™	Nucleic acids	Red	ab108410
Fluorescent	DRAQ7™	Nucleic acids	Red	ab109202
Fluorescent	Nuclear yellow (Hoechst S769121)	Nucleic acids	Yellow/Blue‡	ab138903
Fluorescent	Nuclear Green DCS1	Nucleic acids	Green	ab138905
Fluorescent	Hoechst stain	Nucleic acids	Blue	-

Type	Dye	Target	Color	Product code
Fluorescent	4', 6-diamidino-2-phenylindole (DAPI)	Nucleic acids	Blue	-
Fluorescent	Propidium iodide	Nucleic acids	Red	ab14083
Fluorescent	Fluorophore-tagged phalloidin	Filamentous actin	Fluorophore specific	-

‡ Emission is blue/violet under acidic conditions and yellow under neutral pH conditions

Common special stains and their application

Product name	Application/description	Product code
Acid Fast Bacteria (AFB) Stain Kit	Stains microorganisms - specifically acid-fast bacteria and *Tubercule bacilli*	ab150660
Alcian Blue (pH 1.0) Stain Kit	Stains mucins - acidic mucosubstances stained blue, nuclei pink to red and cytoplasm pale pink (for visualization of strongly sulfated mucosubstances)	ab150661
Alcian Blue (pH 2.5) Stain Kit	Stains mucins - acidic mucosubstances stained blue, nuclei pink to red and cytoplasm pale pink	ab150662
Alizarin Red Stain	Stains calcium – may stain magnesium, manganese, barium, strontium or iron non-specifically depending on concentration	-
Amyloid Stain Kit (Congo Red)	Stains amyloid deposits	ab150663
Colloidal Iron Stain Kit	Stains mucins	ab150664
Combined Eosinophil-Mast Cell Stain Kit	Stains mucins - simultaneously visualize eosinophils and mast cells	ab150665
Copper Stain Kit	Stains copper deposits	ab150666
Elastic Stain Kit (Modified Verhoff's)	Stains connective tissue (e.g., elastin)	ab150667
Fite's Stain Kit	Stains microorganisms (e.g., *Mycobacterium leprae*)	ab150668
Fontana-Masson Stain Kit	Stains melanin and argetaffin granules black	ab150669
Giemsa Stain Kit (May-Grunwald)	Hematologic stain for visualizing cells in hematopoietic tissues and some microorganisms	ab150670
Hydroxystilbamidine (also known as Fluoro-Gold™)	Fluorescent label that can be used as a retrograde enhancer to label neurons (Ex/Em: 385/536 nm)	ab138870
Modified Gomori Methenamine-Silver (GMS) Nitrate Stain Kit	Stains fungi, basement membranes and some opportunistic organisms (e.g., *Pneumocystis carinii*)	ab150671
Golgi Cox Stain Kit	Stains neuronal dendrites and dendritic spines	-

Product name	Application/description	Product code
Gram Stain Kit	Stains microorganisms - differentiates between Gram-positive and Gram-negative bacteria	ab150672
H. pylori Rapid Stain Kit	Stains *Helicobacter pylori*	ab150673
Iron Stain Kit (Prussian Blue)	Stains iron - highly sensitive stain, stains ferric ions in tissue bright blue	ab150674
Luxol Fast Blue Stain Kit	Stains myelinated axons in brain and spinal cord tissue blue, neurons violet and myelin and phospholipids blue/green	ab150675
Methyl Green Pyronin (pH 4.8) Stain Kit	Stains DNA, RNA and mast cell granules	ab150676
Mucicarmine Stain Kit	Stains mucin	ab150677
Nissl Stain	Stains Nissl body in cytoplasm of neurons	-
Oil Red O Stain Kit	Stains lipids and fat	ab150678
Papanicolaou (PAP) Red Stain Kit	Cytology stain - allows differentiation of a variety of cell types in bodily secretions such as gynecological smears	ab150679
Periodic Acid Schiff (PAS) Stain Kit	Stains mucin - stains glycogen, mucin and fungi magenta and nuclei black/blue	ab150680
Picro Sirius Red Stain Kit	Stains connective tissue - specifically collagen	ab150681
Pneumocystis Stain Kit	Stains *Pneumocystis carinii*	ab150682
Phosphotungstic Acid Hematoxylin (PTAH) Kit	Stains connective tissue, specifically collagen, striate muscle and glial fibers	ab150683
Reticulum Stain Kit	Stains reticular fibers in connective tissue	ab150684
Safranin O Stain	Stains mucin, cartilage and mast cell granules	-
Steiner Stain Kit	Stains fungi, *H. pylori*, *L. pneumophila*, and spirochete-infected tissue	ab150685
Sudan Black	Stains lipids and fat blue/black and nuclei red	-
Toluidine Blue Stain	Stains nucleic acids blue and polysaccharides purple. Mast cells are stained dark blue/red purple	-
Trichome Stain Kit (Modified Masson's)	Stains connective tissue	ab150686
Calcium Stain Kit (Modified Von Kossa)	Stains calcium grey to black in histology sections	ab150687
Warthin-Starry Stain Kit	Stains spirochetes, *H. pylori*, *L. pneumophila* and cat scratch fever bacteria	ab150688

Mounting media

Mounting a tissue sample is essential for preserving the specimen during storage and for enhancing imaging quality (clarity and contrast) during microscropy. Mounting media are also used to adhere a coverslip to a tissue section or cell smear. 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 colored precipitate formed is not soluble in the organic solvents used during mounting of the tissue (e.g., DAB). Aqueous mounting media are generally suitable for all enzymatic label/chromogen combinations and fluorescent labels.

Recommended mounting media for non-fluorescent imaging

Product name	Size	Type	Product code
ImmunoHistoMount Medium	30 mL	Aqueous	ab104131
ImmunoHistoMount Medium	100 mL	Aqueous	ab104132
ImmunoHistoMount Medium	250 mL	Aqueous	ab104133
ImmunoHistoMount Medium	1 L	Aqueous	ab104134
Limonene Mounting Medium	30 mL	Organic	ab104141
Mounting Medium	125 mL	Aqueous, organic	ab64230

Recommended mounting media for fluorescent imaging

Product name	Size	Type	Product code
Aqueous Mounting Medium	6 mL	Aqueous	ab128982
BrightMount Mounting Medium	25 mL	Aqueous	ab103746
BrightMount Plus Mounting Medium (Anti-fading)	25 mL	Aqueous	ab103748
Fluoroshield Mounting Medium	20 mL	Aqueous	ab104135
Fluoroshield Mounting Medium	100 mL	Aqueous	ab104136
Fluoroshield Mounting Medium	250 mL	Aqueous	ab104137
Fluoroshield Mounting Medium	1 L	Aqueous	ab104138
Fluoroshield Mounting Medium with DAPI	20 mL	Aqueous	ab104139
Fluoroshield Mounting Medium with DAPI	100 mL	Aqueous	ab104140
Fluoroshield Mounting Medium With PI	20 mL	Aqueous	ab104129
Fluoroshield Mounting Medium With PI	100 mL	Aqueous	ab104130
Glycerol mounting medium with DAPI and DABCO™	20 mL, 100 mL	Aqueous	ab188804

IHC controls

It is essential to run controls in IHC staining experiments to support the validity of the staining pattern and to exclude experimental artefacts. A number of different positive and negative controls must be included. Furthermore, detailed record keeping is key to ensuring consistent performance as variation in experimental conditions and the condition of the tissue itself may impact the reproducibility of staining.

Antigen (tissue) controls

Positive control: a section from a tissue known to express the protein of interest. A positive result from the positive control, even if the samples are negative, will indicate that the procedure is working and optimized. It will verify that any negative results are valid.

Negative control: a section from a tissue known not to express the target antigen. This will check for non-specific signal and false positive results.

Endogenous tissue background control: a section from the tissue before applying the primary antibody. 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.

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. This ensures that staining is produced from detection of the antigen by the primary antibody and not by the detection system or the specimen.

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. Any background staining observed with this control should be negligible and distinct from specific staining. This control can be used when working with monoclonal primary antibodies.

Absorption control: tissue is incubated with pre-absorbed antibody instead of the primary antibody, followed by incubation with secondary antibodies and detection reagents. As this control is performed to demonstrate that the antibody binds specifically to the antigen of interest, little or no staining is expected. 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 due to non-specific interactions between the protein and the tissue.

Tissue slides

Commercially available tissue slides are often used as control tissues in IHC experiments. We offer over 240 tissue slides for:

- Comparison of gene or protein expression in different tissues
- Use as positive and negative staining controls for antibody validation
- DNA or RNA isolation

The slides are suitable for use in IHC, *in situ* hybridization (ISH) and *in situ* PCR assays. They are prepared from both normal and diseased organs, such as spleen, kidney, angioma and Alzheimer's disease brain, and originate from human, mouse or rat. The tissue sections are FFPE and are approximately 5 µm thick. Documentation relating to tissue clinical history is available from our Scientific Support team.

Further information can be found online at www.abcam.com/slides

Tissue microarrays

A tissue microarray (TMA) consists of a histology slide on which representative tissue samples / cores from a selection of different cases are assembled. For investigations requiring a large number of histological sections, TMAs offer several advantages over the use of serial sections, including:

- Better utilization of tissue sections, particularly when these are a scarce resource
- Improved experimental consistency, as identical experimental conditions are used for each core
- Decreased reagent use and associated cost savings
- Options for analysis at the DNA, RNA or protein level (using FISH, mRNA ISH and IHC, respectively)

We offer tissue microarrays containing up to 228 tissue samples per slide from human, mouse and rat tissues. Our portfolio covers a wide range of organs and disease states. Control cores, pathology and disease states are provided where relevant. All tissues were fixed in 10% NBF for 24 to 48 hours. All of our tissue microarrays:

- Are freshly cut (sectioned upon receipt of an order)
- Homogenous for clear analysis of morphology
- Demonstrate low specimen loss (>90% core retention rates)

Documentation relating to tissue clinical history is available from our Scientific Support team.

Further information can be found online at www.abcam.com/TMA

Troubleshooting IHC experiments

The common problems that are encountered during IHC and possible causes and solutions are described in this section.

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 not washed enough, fixative still present.
- Wash extensively in PBS between all steps.

Endogenous peroxidases are active.
- Use enzyme inhibitors i.e., levamisol (2 mM) for AP or H_2O_2 (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 prior to incubating with the substrate, then wash sections/cells in Tris buffer.

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 H_2O_2 (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 against a different species than your tissue.
- Use a commercially available kit with proprietary reagents (e.g., our Mouse on Mouse Polymer IHC kit (ab127055)).

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.

IHC Worksheet

(Photocopy this worksheet to help planning your experiments)

Sample No.	Tissue / cell (type, species, disease state, format)	Fixation (buffer, concentration, temperature, duration)	Type of antigen retrieval (if required)	Antigen retrieval (buffer, pH, composition, duration, temperature)	Blocking step(s) (composition, duration, temperature)

IHC Worksheet

(Photocopy this worksheet to help planning your experiments)

Sample No.	Primary antibody (diluent, concentration, duration, temperature)	Detection system (type, concentration, duration, temperature, label, chromogen)	Mounting media	Additional notes

Notes

Notes