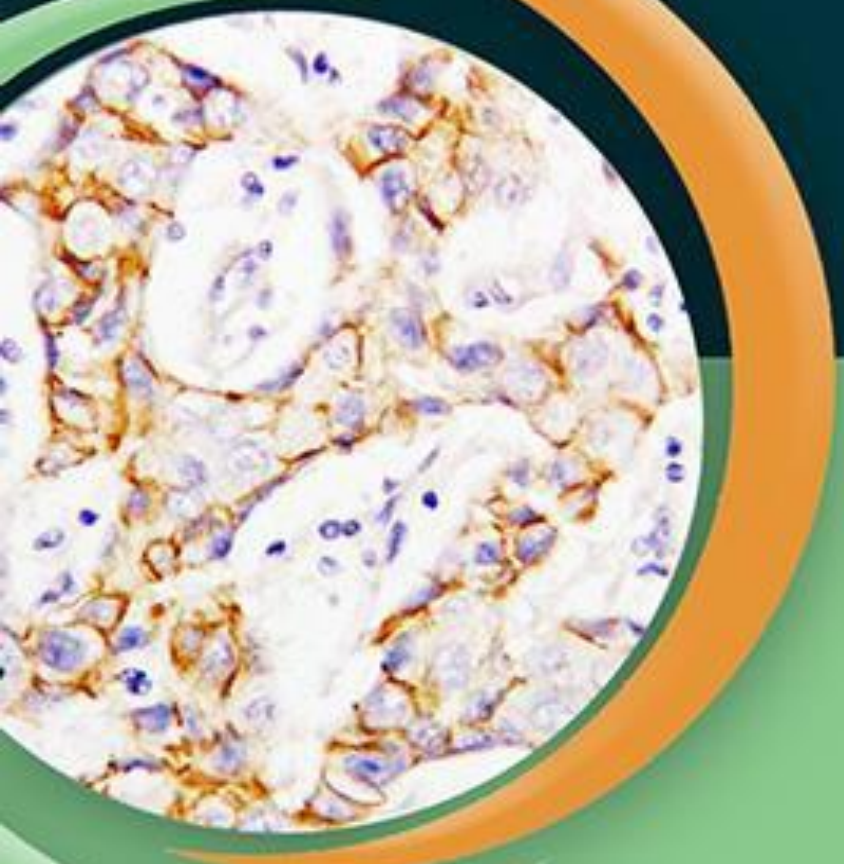
BOSTER

Antibody and ELISA Experts

How to BECOME AN IHC EXPERT in 4 days



IHC 101

A must-read guide for staining slides that everyone wants to read

- Choose the right antibody
- Principle of fixation and tissue sectioning
- HIER vs. PIER
- Step-by-step IHC and ICCF/IF protocols
- Total solutions for troubleshooting weak staining

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Introduction

Immunohistochemistry (IHC) is a method that combines biochemical, histological and immunological techniques into a simple but powerful assay for protein detection. IHC provides valuable information as it visualizes the distribution and localization of specific cellular components within cells and in proper tissue context.

IHC detects antigens or haptens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. The antibody-antigen binding can be visualized in different manners. Enzymes, such as Horseradish Peroxidase (HRP) or Alkaline Phosphatase (AP), are commonly used to catalyze a color-producing reaction. There are numerous IHC methods that can be used to localize antigens. The method selected should include consideration of parameters such as the specimen types and assay sensitivity.

Although ICH does not provide quantitative data as with such assays as Western Blot (WB) or ELISA, IHC can provide a visual context to explain results obtained in quantitative assays. For example, WBs may reveal that the protein of interest is not present in the sample or is only present at very low levels. IHC, in turn, may point to possible underlying causes to explain the lower expression of protein. In many cases, the expression of protein is changed because of altered cellular processes.

For instance, tumor necrosis factor alpha (TNF-alpha) is a cytokine capable of eliciting a wide array of biological responses, mediated by a membrane receptor TNFR1 (Boster catalog# PA1210). TNF-alpha has been shown to be upregulated following stroke and is believed to be part of the acute inflammatory process.

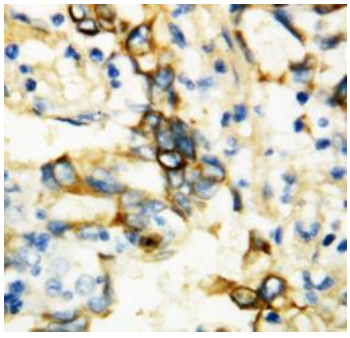

Figure 1. Anti-TNF Receptor I antibody (Boster Catalog# PA1210) in Human Mammary Paraffin-Embedded Tissue by IHC.

A well-known example of demonstrating the utility of IHC assays comes from a diagnostic screen for cancer. The assay was developed on the basis of research, revealing that a "tumor suppressor" gene associated with cell cycle regulation (p27Kip1, "cell cycle inhibitor protein", Boster Catalog# MA1076) is inactivated in many cancers and, in turn, cell proliferation is promoted. IHC and other experimental techniques have demonstrated that, depending on the type of cancer involved, this inactivation can occur via degradation, impaired synthesis or improper localization.

Many scientists doing basic research use IHC to demonstrate altered localization, translocation, processing, trafficking and targeting of their proteins of interest within cells and tissues. For example, some proteins translocate between different cellular compartments in response to certain stimuli. Bcl-2, for instance, translocates from the cytoplasm to the nucleus in response to ischemia following stroke. Nuclear translocation of the protein has been convincingly demonstrated by IHC (in combination with other experiments) before and after cerebral hypoxia–ischemia is induced.

Design Your IHC Experiment

There are a number of important considerations that must be taken into account and variables that must be optimized in order to consistently obtain robust and reproducible results from the IHC experiments. Selection of proper antibodies, method of tissue fixation used, epitope rescue, blocking procedure, antibody concentrations and control must all be considered in the design of the experiment. In the following guide, we discuss these considerations and suggest sample protocols to help in the design of your IHC experiments.

1. Choose The Right Antibody

Table 1. Factors Involved in Selecting Proper IHC Antibodies.

Variable	Options	Considerations	
Antibody Type	Monoclonal	Advantages • Mouse or rabbit hybridoma • Good consistency	Disadvantages • Lower affinity • False negatives common
	Polyclonal	Advantages • Several different species	Disadvantages • More non-specific reactivity • Affinity may vary between lots
Antibody Target	Entire Molecule	• Depending on location of target antigen e.g., If C-terminus is embedded in a membrane, it is not a good choice for a target.	
	C-Terminus		
	N-Terminus		

2. Monoclonal vs. Polyclonal

Monoclonal antibodies are created with a single B-cell clone from one animal. To generate large amounts of homogenous antibodies, these clones are fused with a myeloma (B-cell cancer) to create an immortal cell called a hybridoma which produces immunoglobulins specific for a single epitope. Researchers like monoclonal antibodies because they are easy to standardize due to consistency between batches. Although targeting of a single epitope means less cross-reactivity with other proteins, this feature can also mean that the antibody is too specific, resulting in false negatives.

On the other hand, polyclonal antibodies are created using multiple B-cell clones of the animal which

results in a heterogeneous mix of antibodies that target various epitopes of the same antigen. As a result, polyclonal antibodies are more forgiving of changes in target proteins that can easily result from the fixation process. The fact that they recognize multiple epitopes also means that they can amplify signal in samples in which the protein of interest is expressed at low levels. This feature also makes polyclonal antibodies the antibodies of choice when dealing with denatured proteins.

Further, while monoclonal antibodies are generated from mouse and rabbit hybridomas, polyclonal antibodies are raised in several different species, including not only mice and rabbits but also rats, goats, sheep, guinea pigs, chickens and more.

The primary antibody should be raised in a host species that is as different from the source of your samples as possible. Otherwise, IgG secondary antibodies will bind to all the endogenous IgG in the tissue, leading to high background.

3. Region of Targeted Protein

Antibodies are generated by using an array of immunogenic substances to induce an immune response in a host animal. These immunogenic substances may include full-length proteins, protein fragments, peptides and whole organisms such as bacteria or cells. If the target epitope is a protein fragment or a specific isoform or region of a full-length protein, the experimenter must be sure to choose an antibody against an epitope that is identical to or contained within the fragment or region. For example, if the target is a membrane protein, then the appropriate antibody should target the protein's extracellular domain.

Some protein families contain a conserved motif made up of a sequence of amino acids. Small variations within this motif can be exploited to differentiate between subtypes of proteins within the family. An example of this strategy is the Glucose Transporter 5 (GLUT5), which can be detected by targeting a 19 amino acid sequence found in the middle of the receptor.

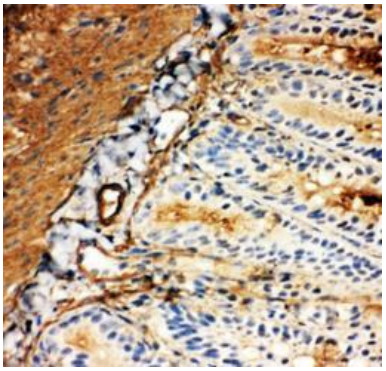

Figure 2. Anti-Glucose Transporter 5 stained by IHC on rat intestinal (frozen) tissue with Boster antibody (Catalog# PA1737).

Principle

In order to be able to use certain antibodies, samples must be processed or fixed in a very specific manner. For example, many antibodies only recognize proteins that have been reduced and denatured, while others only detect protein epitopes when the proteins are in their native, folded conformation. Some antibodies should only be used with unfixed, frozen tissue. Many antibodies cannot bind to their target epitopes in formalin-fixed, paraffin-embedded tissues unless an antigen retrieval step is included to reverse the cross-links that result from fixation.

If your protein of interest is intracellular, cells must be permeabilized with solvents or detergents to allow antibodies access to the interior of the cell. Detergents are the most popular method for permeabilizing cells. However, because they disrupt the membrane, detergents are not appropriate for cytoskeletal antigens or low molecular-weight targets such as viral antigens and some enzymes. Note that permeabilization is necessary for ICC. For IHC (Paraffin and frozen sections), the tissue is usually sliced thinly, and thus permeabilization is not required.

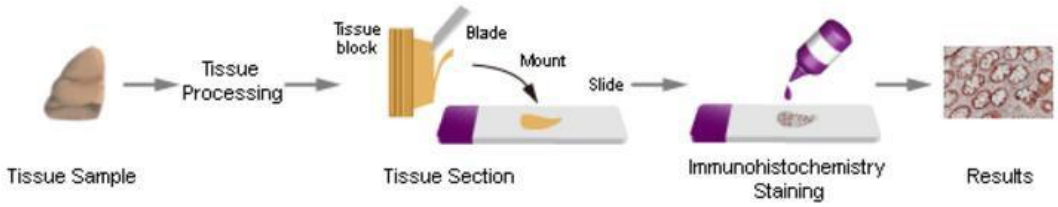

Figure 3. IHC Workflow from Sample to Results.

In this handbook, the following topics will be covered:

1. Fixation
2. Tissue Sectioning
3. Paraffin Embedding
4. Inactivation and Blocking
5. Antigen Retrieval
6. Detection
7. Chromogens, Counterstains and Mounting Media

1. Fixation

Fixation preserves morphology, prevents tissue degradation, ensures antigenicity and helps protect cells from damage during the strenuous tissue preparation process. The reproducibility and quality of IHC results are dependent on fixation times, temperatures, pH and buffer composition — all of which must be carefully optimized and standardized by the experimenter.

(A) Purposes

- Keep cell sharp and tissue shape to prevent postmortem autolysis, putridness, endogenic and

exogenic enzyme activity

- Maintain cell structure and position by preventing antigen diffusion through transfer of protein, fat, sugar and enzymes of cell into insoluble substances
- Precipitate and curdle materials in tissue to produce different refraction
- Indurate tissues to enhance working with glass slides
- Prevent cell from shrinking and swelling
- Give color to clarify tissues by different affinity to coloring agent

(B)) Selection of Fixing Solution

Below is a list of commonly used fixing solutions. You may need to test whether a specific type of solution is appropriate for your detected antigens because there is no standard fixing solution for different kinds of antigen immobilization.

(i) Acetone and Alcohol

These two types of solutions, which are primary fixing solutions, play a role of precipitating sugars and fat as well as maintain the immunologic competence.

- Alcohol is ineffective to maintain low molecular weight protein, polypeptide and cytoplasmic proteins. However, it can be mixed with glacial acetic acid, ethyl ether, chloroform and formaldehyde.
- Acetone is often used for frozen tissue and cytological smears because it has a strong penetrability and dehydration property.

(ii) Aldehyde

It is a di-functional cross-linking agent which is widely used due to its strong penetrability, low contractibility and low background. It helps keep the cross-linking between tissues and maintain antigen.

- Formalin (10% neutral buffered) is the most widely used
- 4% paraformaldehyde is better than formaldehyde
- Bouin's solution (containing picric acid) is the most widely used in histology and pathology
- Zamboni's solution is applied to light and electron microscopic immunocytochemistry and is better than formaldehyde in ultrastructural organization maintenance

(iii) Non-Aldehyde

- Carbodiimide, dimethylacetamide, dimethyl-suberimidate, para-benzoquinone are widely used in tissue fixation of peptide hormones
- These fixation agents are better mixed with glutaric dialdehyde or paraformaldehyde

In recent years, a new type of formaldehyde-free fixing solution has become available. With low toxicity and degradable chemical agent, this solution has gained a broad popularity in IHC, regular pathological examinations and molecular pathology detections due to the use of non-protein cross linking, strong DNA/RNA preservation *and* absence of cell vacuole, tissue shrinkage and pyknosis.

(C) Method

(i) Fixation by Perfusion

The best way to preserve tissue morphology and target protein antigenicity is to replace the animal's entire systemic blood volume by fixative with vascular perfusion. This is followed by perfusion with sucrose solution. It is important to remember that fixation can cause cross-linking which masks epitopes.

(ii) Fixation by Immersion

It is sometimes impractical or inappropriate to fix tissues by perfusate. An alternative method is to immerse tissues in fixative solution (at 4°C if needed) for a specified period which is determined by the antigen stability and type of fixing solution used. Biopsy and surgical specimens as well as other non-irrigation tissues commonly employ this fixation method.

(iii) Snap Freezing

It is not always possible or appropriate to fix tissues before sectioning. For example, phosphorylation-dependent epitopes have been shown to translocate from the membrane to the cytoplasm following fixation with formaldehyde. For cases in which standard fixation techniques cannot be used or fixation will be carried out later, tissue can be snap frozen and kept at -70°C until use.

Exercise caution when fixating tissues

- Do not over-fix the tissues
- Keep the tissues fresh after fixation
- Use enough fixing solution and wash it off completely after fixation
- Use tissues of size less than 2 cm × 1.5 cm × 0.3 cm (Thickness < 0.3 cm)

2. Tissue Sectioning

(A) Slide Pre-Treatment

The following pre-treatment procedure is designed to prevent peeling caused by elevated temperature, high pressure, radiation and other factors.

(i) Microscopic Slide

Due to the oil attached on surface, a new slide should be dipped into cleaning solution for 12 to 24 hours. After washing the slide more than 5X in distilled water, dip it into 95% alcohol for 2 hours followed by drying with simple wiping or in an infrared oven. Pay attention to avoid scratching the slide during washing.

Note: Microscopic slide for IHC is required to be 5 μm. However, the slide for nervous tissue should be 20-100 μm to enhance the tracking of never fiber direction.

(ii) Cell Samples

(a) Adherent Cells

- Cell Climbing: Grow adherent cells on multi-aperture culture plates with coverslip*, culture vessels or chamber slide
- Direct Cell Culture: Culture adherent cells directly on culture vessels or multi-aperture culture plates

* The coverslip pre-treatment procedure is very similar to the one for the microscopic slide except that dipping and cleaning should be completed in 2 hours because the coverslip is much thinner)

(b) Non-Adherent Cells

- Cell Smear: Adhere non-adherent cells on coverslip with chemical bond
- Eccentric Cell Smears: Adhere non-adherent cells on culture vessels by cell micro-centrifuge

(iii) Tissue Section Mounting

- Dilute 3-Amino Propyl Tri-ethoxy Silane (APES) by acetone
- Coat the microscopic slide with APES
- Apply tissue on the coated slide followed by adding a drop of mounting medium (Glycerol Gelatin) on the tissue
- Hold the coverslip at 45°, allowing the drop to spread along the edge of the slip
- Slowly cover the tissue entirely with the coverslip
- Incubate the slide at 80°C for 1 hour if there is an adhering problem with a tissue section

(B) Tissue Section Types

Tissue samples are typically taken from specimens of various sources: biopsy, surgery, animal model and autopsy. The first three types of specimens give fresh tissues while the last one (autopsy) is taken after an animal has died for two hours which is more or less a postmortem autolysis. As antigens may denature, disappear and diffuse, autopsy specimen should be fixated as soon as possible so as not to influence its label.

(i) Frozen

The most important feature for this type of tissue section is to keep antigen's immune-competence completely, especially for the cell surface antigen. Both fresh and fixed tissues can be processed as frozen tissues. However, the tissues must be dried (or primary fixed) and stored at low temperature.

(ii) Paraffin-Embedded

Paraffin-embedded tissue section is normally sliced by a rotary microtome to give a thickness of 2-7 μm. With proper treatment, the section reveals clear tissue structure and exact antigen location to enable high medical-value pathology researches and retrospective studies. This section type can be stored at 4°C for long term use.

Exercise caution when collecting, fixating and sectioning the samples

- Use sharp knife and scissors to avoid extrusion damage
- Cutter should be flat, small and thin (Normal size is 1.0 cm × 1.0 cm × 0.2 cm)
- Eliminate fat tissue and calcification zone
- Collect samples from live animals and fix samples immediately after wash
- Choose diseased instead of necrotic region
- Choose normal tissue as control if necessary
- Make paraffin-embedded tissue or frozen tissue immediately after sectioning or store the tissues in liquid nitrogen container or refrigerator at -70°C

3. Paraffin Embedding

Five major steps are involved in paraffin embedding: fixation, dehydration, transparentizing, immersion and embedding.

(A) Fixation

Please refer to the Fixation section described above.

(B) Dehydration

This step removes water completely, creates a condition for the next step and hardens the tissue of interest. The dehydrating agents describes below are completely miscible with water and can be prepared in different volumetric ratios with water.

(i) Ethanol

As the most commonly used dehydrating agent, ethanol has a strong water separation and tissue hardening capability. However, since ethanol has strong penetration and contractility, its concentration should be progressively increased to avoid tissue excessive shrinking.

(ii) Acetone

As a usual substitute for alcohol, acetone acts as both a fixing solution and fast dehydrating agent. Pay attention to the dehydrating time with acetone as it tends to over harden tissues.

(C) Transparentizing

After dehydration, the tissue of interest requires a transparentizing step because the dehydrating agent used in the previous step is immiscible with the paraffin from one of the subsequent steps. The addition of transparent reagent helps paraffin absorb into the tissue. Common transparent reagents are:

(i) Xylene

As the most widely used transparent reagent, xylene is miscible with both ethanol and acetone, and it acts as a fusing agent for paraffin wax. Since xylene has a strong and fast contractility to tissue, the tissue should not be immersed for an extended time period or it will be over crisp and too hard.

(ii) Benzene and Toluene

These reagents are similar to xylene. However, they have weak and slow contractility to tissue, and therefore the tissue can be immersed in these reagents for a longer time. Note that benzene and toluene have high toxicity and must be handled with care.

(iii) Chloroform

Compared to xylene, benzene and toluene, chloroform is a much gentler reagent. However, it has a small refractive index, and the tissue should thus be immersed in chloroform for a longer time than the other transparent agents in order to guarantee complete penetration.

(iv) Cedar Oil

Due to minimal sclerification created by cedar oil, it is an appropriate transparent agent for fine and soft tissues. Super hard tissues (e.g. skin tissue) and dense fibrous tissue are also easier to be sectioned after immersing in cedar oil. However, this oil is not useful for other common tissue sections because of its high concentration and weak penetrability.

In recent years, a new type of environment-friendly transparent agent has appeared in market. Instead of aromatic compound, the main components of this new reagent is alkanes, and it can be used to replace xylene.

(D) Immersion

After transparentizing, the tissue can be immersed in molten paraffin wax so that it adsorbs the wax-substituting transparent agent. Based upon the melting point of wax, immersion should be performed at 54-64°C.

(E) Embedding

This is a process of treating the tissue in a paraffin box so that the paraffin wax cools down and solidifies. The treatment conditions (using ethanol and xylene as an example) are shown in the table below. After cooling is completed, the tissue will be ready for sectioning and suitable for storage.

Table 2. Paraffin Embedding Treatment Conditions.

Step	Reagent	Time (Hours)
1	75% Ethanol	0.5 to 2
2	85% Ethanol	0.5 to 2
3	95% Ethanol	2
4	95% Ethanol	2
5	95% Ethanol	2
6	100% Ethanol	0.5 to 1
7	100% Ethanol	0.5 to 1
8	100% Ethanol	0.5 to 1
9	Xylene	0.25
10	Xylene	0.25
11	Xylene	0.25
12	Paraffin Wax	0.5
13	Paraffin Wax	1 to 2
14	Paraffin Wax	1 to 2

4. Inactivation and Blocking

(A) Inactivation

When either the horseradish peroxidase (HRP) or alkaline-phosphatase (AP) system is applied for IHC, activation of endogenous enzymes should be blocked or inhibited to avoid producing non-specific binding.

(i) Endogenous HRP Inactivation

- Incubate the paraffin embedded section in 3% H_2O_2 for 10 min
- Incubate the frozen section or cell section in solution composed of methanol and 3% H_2O_2 (v/v: 4:1) for 30 min

(ii) Endogenous AP Inactivation

- Incubate the sample section in 0.1 mM Levamisole
- Note: Levamisole cannot inhibit the AP activation of endogenous intestine tissue

(B) Blocking

Residual sites on the tissue section may bind to secondary antibody and produce follow-up false positive results. Therefore, serum from the same species as the secondary antibody is commonly used for blocking. Animal's autoantibody in the serum can bind to the sites in advance. Blocking should be done at room temperature for 10-30 min (avoid excessive blocking).

5. Antigen Retrieval

Formaldehyde fixation usually generates methylene bridges which cross-link proteins and therefore mask the epitope of interest. It is essential to unmask the antibody epitopes in order to allow the antibodies to bind, either by heat (Heat Induced Epitope Retrieval: HIER) or enzymatic digestion (Proteolytic Induced Epitope Retrieval: PIER). To find the optimal antigen recovery method, we suggest that you test both HIER and PIER methods, compare their results and optimize the method as needed.

(A) HIER

The HIER method can be implemented by microwave, high pressure or water bath. It breaks the methylene bridges and exposes the epitope to allow the antibodies to bind by continuously heating. Comparing to PIER, HIER has a gentler experimental condition in which users have more control over the experimental parameters. However, the pH and buffers for HIER must be optimized. The following antigen retrieval reagent is required:

- 0.01 M citrate buffer solution (pH 6.0)
- 0.01 M PBS buffer (pH7.0)
- 0.05 M EDTA (pH 8.0)
- 0.05 M Tris-EDTA (pH 9.0)
- 0.05 M Tris-HCl (pH 1~12)

(i) Microwave Method

- Place the sample section into a microwaveable vessel where antigen retrieval reagent is present
- Place the vessel inside a microwave oven
- Apply microwave radiation to the sample for 5-20 min

(ii) High Pressure Method

- Place the sample section into an appropriate vessel where antigen retrieval reagent is present
- Place the vessel inside a pressure cooker
- Turn on the cooker and heat the sample until it boils
- Once boiling starts, turn off the cooker after the sample is allowed to reach full pressure for 1-4 min

(iii) Water Bath Method

- Place the sample section into an appropriate vessel where antigen retrieval reagent is present
- Place the vessel and thermometer inside a water bath chamber
- Heat the sample to 92°C in the chamber
- Remove the sample from the chamber after it is heated at 92°C for 20-40 min

Notes

- The temperature and time should be properly controlled for the antigen retrieval methods described above.
- To avoid original protein structure restoring, do not cool the sample section by taking it out of the buffer solution.
- The higher the temperature, the shorter the heating time (vice versa).

(B) PIER

Epitope can be exposed by incubation with proteases which can break the methylene bridges. The choice for digestion enzymes depends on the antigenic components. Pepsin and bromelin are used for retrieving antigens in intercellular substance. Other enzymes can be used for intracellular antigen exposure. PIER is suitable for retrieving more difficult epitopes while the pH for incubation is usually known. However, PIER is a harsher method and can damage tissue morphology.

Table 3. Digestion Enzymes for PIER.

Enzyme	Working Concentration	Digestion Condition
Trypsin	0.05% to 0.1%	37°C (10 to 40 min)*
Proteinase K	20 μg/mL	37°C (20 min)
Pepsin	0.4%	37°C (30 to 180 min)

* The reaction time can be increased for certain worn-out tissues. Fresh trypsin solution should be prepared with pH adjusted to 7.6 and used at 37°C.

6. Detection

IHC detection methods vary and are based on the nature of analyze reporting and binding chemistry, among other factors. Three methods are described here: immunofluorescence (IF), Enzymatic and Affinity.

(A) Immunofluorescence Method

Coons and co-workers developed the IF technique in 1941. This technique is used for the rapid identification of an antigen by exposing it to known antibodies labeled with the fluorescent dye (i.e., fluorochrome) which produces light when excited by a laser (e.g. argon-ion laser). Specific antibody binding can be determined by the production of characteristic visible light and detected by a fluorescence microscope. Tables 1 and 2 show some of the common fluorechromes and their corresponding excitation (λex) and emission wavelengths (λem) for nuclear staining and IF, respectively.

Table 4: Common Fluorochromes for Nuclear Staining.

Fluorochrome	λex (nm)	λem (nm)	Color
AO	405	530 → 640	Yellowish (Green → Orange)
DAPI	358	461	Blue
EB	488	610	Red
PI	488	620	Red
Hoechst 33258	352	461	Blue
Hoechst 33342	352	461	Blue

Table 5: Common Fluorochromes for IF Labeling.

Fluorochrome	λex (nm)	λem (nm)	Color
Alexa 488	488	497 to 643	Green
Alexa 546	530/545	610/675	Red
Alexa 647	650	668	Red
APC	650	660	Red
B-PE	546, 565	575	Orange, Red
Cy3	554	570	Red
FITC	495	525	Green
RB200	570	596	Orange
R-PE	480, 546, 565	578	Orange, Red
Texas Red	596	620	Red
TRITC	552	570	Red

(i) Principle

The indirect staining process involves three steps

- Primary antibody binds specifically to target antigen
- Secondary antibody labeled with fluorophore binds to primary antibody
- Fluorophore is detected via microscopy

(ii) Tips: Operations of Fluorescence Microscope

- Operate the microscope according to the manual
- Turn on the mercury lamp for 5-15 min to stabilize the light source before use
- Wear protective glasses when adjusting light source to avoid harmful ultraviolet rays to eyes
- Intensity of high pressure mercury lamp will drop if the lamp is used for more than 90 min (Typically, the lamp is continuously used for 1-2 hours)
- Photo-bleaching occurs if the sample is illuminated by high pressure mercury lamp for more than 3 min (Note: The sample is generally observed within one hour after fluorescence staining)
- Observe the samples intensively to save time as light source is limited
- Re-start the light source after turning it off for 30 min or longer
- Avoid using the light source several times during one day
- Observe samples immediately after staining
- There are four levels for fluorescence intensity:
  －: Non or weakly visible autofluorescence
  ＋: Clearly visible fluorescence
  ＋＋: Brightly visible fluorescence
  ＋＋＋: Dazzling visible fluorescence

(iii) Counterstaining and Stained Sample Storage

(a) Nucleus Counterstaining

After fluorescence staining, counterstain should be carried out to make morphological structure of cells and tissues well defined *and* specific fluorescence more easily visible. Some of the counterstaining fluorochromes are:

- DAPI: classic blue counterstain which is used extensively for nucleus and chromosome staining (DAPI binds selectively to dsDNA without background staining in cytoplasm; DAPI has semi-permeability to living cells and can be used to stain fixed cells and/or tissue sections)
- Hoechst 33342: primary counterstain which is used against yellow fluorescence
- Propidium iodide: primary counterstain which is used for nucleus and chromosome staining against yellow/red fluorescence

(b) Stained Sample Storage

After staining, the samples should be observed and imaged immediately under a fluorescence microscope. They should be mounted in buffered glycerol medium and stored at 4°C for less than one week if the image is not taken immediately. If anti-fluorescence decay medium is applied to the sample, fluorescence signal may not decay significantly within one month.

(B) Enzymatic Method

The enzymatic IHC technique was introduced by Nakane and Pierce in 1967. It identifies antigens of interest by exploiting the principle of antibodies binding specifically to antigens. An enzyme label is reacted with a substrate to yield an intensely colored product that can be analyzed. The enzymatic technique was developed with a similar principle to the IF technique but the two are different as an enzyme is used to label the antibody for the enzymatic method. The advantages of enzymatic IHC over IF IHC are:

- Fluorescence microscope is not required
- Accurate antigen location is enabled with better contrast ratio
- Stained samples can be stored for a long time
- Haematoxylin can be used as counterstain which enhances study of tissue morphology
- End-product color can be easily identified and observed by light microscope (and also by electron microscopy due to high electron density)
- Double and multiple stains can be implemented

(i) Labeled-Enzyme Antibody

For this method, the antibody used for antigen detection has been labeled with the enzyme before the reaction. After reacting with the targeted antigen, the labeled antigen forms an antigen-antibody complex where the enzyme catalyzes a substrate to yield an insoluble colored product. Subsequently, the product can be analyzed by a light microscope or electron microscope. The labeled-enzyme approach can be done by direct or indirect detections.

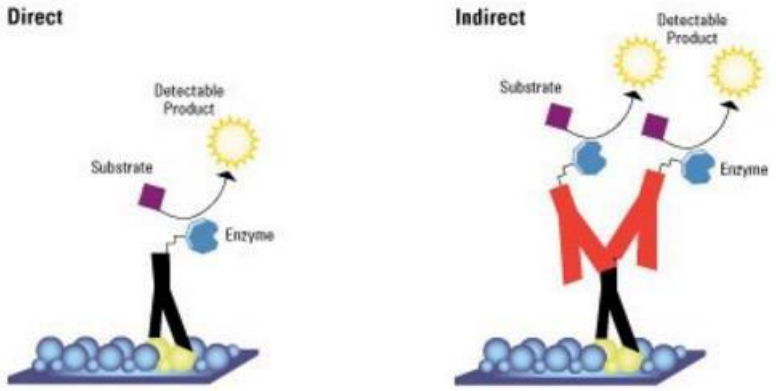

Figure 4. Direct vs. Indirect IHC Detection Methods.

(a) Direct Detection

The direct method is a one-step staining method which involves a labeled antibody (e.g. HRP-conjugated antibody) reacting directly with the antigen of interest. The antigen-antibody-HRP complex is then allowed to react with a DAB substrate for staining.

While the direct method is simple, rapid and highly-specific, it has low sensitivity and a limited range of primary antibodies that are directly labeled. Despite the shortcomings, the direct method is commonly applied to screen monoclonal antibodies before the large-scale manufacturing process.

(b) Indirect Detection

The indirect method is a two-step process which involves an unlabeled primary antibody (first layer) that binds to the target antigen in the sample and an enzyme-labeled secondary antibody (second layer) that reacts with the primary antibody. The secondary antibody must be raised against the IgG of the animal species in which the primary antibody has been raised. For instance, if the primary antibody is rabbit anti-human IgG, the enzyme labeled secondary antibody could be goat anti-rabbit IgG.

Comparing to the direct detection, the indirect detection has numerous advantages. First of all, only a relatively small number of standard conjugated (labeled) secondary antibody is needed to be generated for the indirect method. For example, a labeled secondary antibody raised against rabbit IgG, which can be purchased "off the shelf," is useful with any primary antibody raised in rabbit. With the direct method, it would be necessary to label each primary antibody for every antigen of interest. Secondly, the indirect method has greater assay sensitivity. Thirdly, various kinds of controls could be designed and applied with indirect detection.

(ii) Unlabeled-Enzyme Antibody

(a) Enzyme Bridge Method

Described by a publication from T.E. Mason and his colleagues in 1969, this method is based on the binding of an enzyme label to a target antigen through the antigen-antibody reactions of an immunoglobulin-enzyme bridge which consists of the following components in order:

- Specific antiserum for the tissue antigen (AnTAn)
- Antiserum against the immune globulin of the species for AnTAn
- Specific antiserum prepared against the enzyme label in the same species as AnTAn
- Enzyme label

(b) Peroxidase-Anti-Peroxidase (PAP) Method

This method involves immunization of a rabbit/goat/rat antibody with a HRP moiety to produce an anti-HRP rabbit/goat/rat antibody which would then bind to another HRP moiety to form a stable polygon. The PAP approach excels due to its high sensitivity and low background for tissue staining.

(C) Affinity Method

The IHC sensitivity can be improved by employing a higher number of enzyme molecules bound to the tissue. In this regard, the multiple binding sites between the avidin and biotinylated antibodies have been exploited for IHC signal amplification. Avidin, an egg white protein, has four binding sites for the low-molecular-weight vitamin biotin to form a large lattice-like complex. Beside avidin, there are other methods which involve streptavidin which is a tetrameric biotin-binding protein that is isolated from *Streptomyces avidinii*. The avidin and streptavidin methods work almost identically as their structures are very similar (they have very little amino acid homology). Avidin-Biotin Peroxidase Complex (ABC) and Labeled Streptavidin Binding (LSB) are the two most widely used affinity methods for amplifying the target antigen signal.

(i) ABC

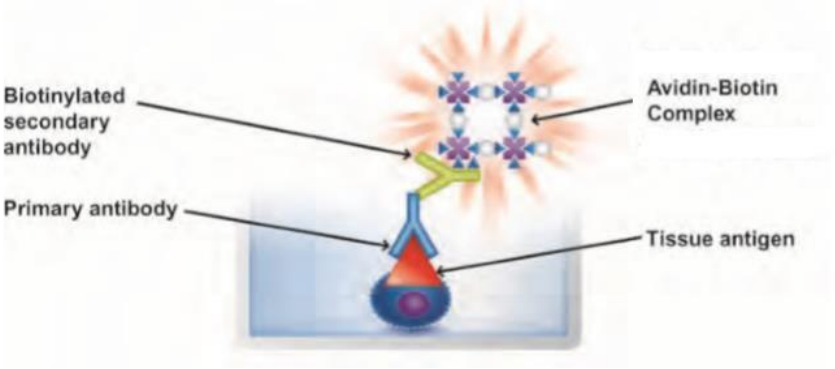

Figure 5. ABC Detection Method.

The method involves four sequential steps

- Incubation of primary antibody with tissue sample to allow binding to target antigen
- Incubation of biotinylated secondary antibody (which has specificity against primary antibody) with tissue sample to allow binding to primary antibody
- Pre-incubation of biotinylated enzyme (HRP or AP) with free avidin to form large ABC complexes (Biotinylated enzyme and avidin are mixed together in a pre-determined ratio to prevent avidin saturation)
- Incubation of the above pre-incubated solution to tissue sample

(ii) LSB

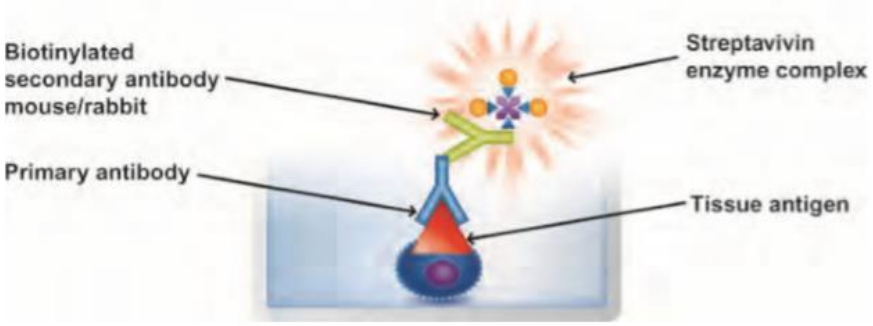

Figure 6. LSB Detection Method.

This method uses an enzyme-labeled streptavidin to detect the bound biotinylated primary antibody on the tissue section. It can also be applied if the complex in the ABC method is too big for tissue penetration. Due to its smaller size, the enzyme-labeled streptavidin is used to enable tissue penetration. The LSB method can be employed to replace the ABC method for the former's ability to improve sensitivity and reduce signal further. The information below describes the general staining procedure.

- Incubation of primary antibody with tissue sample to allow binding to target antigen
- Incubation of biotinylated secondary antibody (which has specificity against primary antibody) with tissue sample to allow binding to primary antibody
- Incubation of streptavidin-enzyme conjugate to tissue sample

7. Chromogens, Counterstains and Mounting Media

(A) Chromogens for HRP

(i) DAB

DAB (3,3'-Diaminobenzidine) is typically used as a signal enhancer in conjunction with the HRP-based immunostaining systems. The dark brown end-product derived from DAB is insoluble in water and alcohol, stable and suitable for long-term storage. In addition, the end-product could be observed under a light microscope or processed with OsO_4 for observation under electron microscopy. Haematoxylin, methyl green and methyl blue are the compatible counterstains. Since DAB may cause skin and bladder cancers, it is advised that personal protective equipment should be used and skin/mucosa should be avoided.

(ii) AEC

After staining with AEC (3-Amino-9-Ethylcarbazole), the positive area on tissue section changes to dark red. The end-product derived from AEC is soluble in organic solvent and cannot be stored on a long-term basis. Similar to DAB, haematoxylin, methyl green and methyl blue are some of the suitable counterstains for AEC. Glycerin gelatin should be used as the

AEC mounting medium.

(B) Chromogens for AP

(i) BCIP/NBT

Used in conjunction, BCIP (5-Bromo-4-Chloro-3-Indolyl-Phosphate)/NBT (Nitro Blue Tetrazolium) is a widely accepted chromogenic substrate used in the AP-based immunostaining systems. After exposing to AP, the substrate changes to bluish violet or black violet. The end-product derived from BCIP/NBT is insoluble in alcohol. Nuclear fast red and brilliant green are the suitable counterstains for BCIP/NBT.

(ii) Fast Red TR Salt

Fast Red is also used for the colorimetric detection of AP. Its end-product has a rose color and is soluble in alcohol. These counterstains are used for the Fast Red chromogens: methyl green, brilliant green and soluble haematoxylin.

(C) Counterstains

After staining the target antigen by IHC, a secondary stain is usually applied to provide contrast that helps the primary stain more distinct. While many of these stains show specificity for discrete antigens or cellular compartments, other stains will deliver the staining of a whole cell. Some of the most common counterstains are described as follows:

(i) Haematoxylin

Haematoxylin, a natural dye which is extracted from the heartwood of the logwood tree, is used for cell nucleus staining. Differentiation refers to the process of using reagents (e.g. 1% hydrochloric acid HCl and alcohol) to remove the color caused by overstaining or non-specific staining on sample tissues. After running nucleolar staining (in aluminum haematoxylin) and differentiation (in HCl and alcohol), the tissue section is transferred from an acid solution to an alkaline solution (e.g. ammonia water and disodium hydrogen phosphate solution). During this process, the section will change from red brown into blue. This procedure is known as bluing.

Haematoxylin is sub-categorized into Mayer's Haematoxylin and Harris Haematoxylin.

(a) Mayer's Haematoxylin is reddish violet and is valued for several properties: low staining time, no perception and metal membrane as well as no post-staining differentiation.

(b) Harris Haematoxylin is purple red, widely used in H&E staining and has these advantages: fast staining, bright color, clear nucleolar stains and well defined tissue morphology. Although metallic oxide may float on the Harris haematoxylin solution after a long period of time, filter is unnecessary before use as no perception will appear. Differentiation and

bluing should be carried out after staining with Harris Haematoxylin.

(ii) Methyl Green

Methyl green consists of metallic green microcrystals or bright green powders. It becomes bluish green when dissolved in water. This basic dye can be easily bounded with highly polymerized DNA and changes the nucleus to green. Counterstain with methyl green takes 2 to 5 min which should be followed by washing the sample, dehydration and mounting.

(iii) Nuclear Fast Red

This counterstain will change the nucleus to red after applying to the tissue section for 2 to 5 min.

(D) Mounting Media

A mounting medium may be used to attach a coverslip or may itself be used to replace the coverslip. Generally, the medium selection depends on a few factors including the chemical compatibility with chromogen and counterstain as well as the preservation period.

(i) Neutral Mounting Medium

It usually refers to an oily substance with pH 7.0 such as neutral gum (resin). Before mounting, the sample should be treated with dimethyl benzene, transparent and dehydrated for long-term storage sections.

(ii) Water-Soluble Mounting Medium

Popularly used in IF staining for short-term storage sections, this mounting medium usually consists of 50% glycerol.

The table below summarizes the choice of mounting medium among different enzymes, chromogens and counterstains.

Table 6. Chromogens, Counterstains and Mounting Media for HRP and AP Enzymatic Systems.

Enzyme	Chromogen	Counterstain	Mounting Medium
HRP	DAB	Haematoxylin, Methyl Green, Methyl Blue	Neutral
HRP	AEC	Haematoxylin, Methyl Blue	Water Soluble
AP	BCIP/NBT	Nuclear Fast Red, Brilliant Green	Neutral
AP	Fast Red	Haematoxylin, Methyl Green, Brilliant Green	Water Soluble

8. Controls

Every IHC experiment must include positive and negative controls. However, several other types of controls can be considered based on the type of experiment that you plan to work on.

(A) Positive Controls

A positive control validates the staining of your sample and confirms that the assay is working correctly. It will also help optimize the conditions for future experiments. To identify suitable positive controls, a good starting place is to check the antibody datasheet any of these may be used as your positive control. You can also check the Swiss-Prot or Omnigene database links on your antibody's datasheet. The databases often include a list of tissues that express your protein interest which can be used for positive controls. In addition, the following resources are helpful:

- GeneCards: It usually provides with relative levels of expression in various tissues
- Human Protein Atlas: Its protein detection database has different tissue types, cancers, and cell lines
- Pubmed: The literature search may give you ideas as to which tissues and cells express the protein of interest

(B) Negative Controls

Negative controls, on the other hand, reveal non-specific binding and false positive results. Obviously, negative controls should not express your protein of interest. A common negative control tissues are knock down (KD) or knock out (KO) tissue samples.

(C) No Primary Controls

For this type of control, primary antibody is not added to the sample. These controls are indicative of non-specific binding or false positives that can result from non-specific binding of the secondary antibody. Antibody dilution buffer with no antibody added is incubated on the same sample under identical conditions.

(D) Isotype Controls

Isotype controls are antibodies of the same isotype (e.g. IgG2, IgY), clonality, conjugate and host species as the primary antibody, targeting a molecule that is not present in your sample. For example, the target is generally a chemical or a non-mammalian protein. If used, incubate your samples with the isotype control antibody rather than the specific primary antibody (The concentrations and experimental conditions between the primary and isotype control antibodies are the same).

(E) Endogenous Control for Transfected Cell Lines

If your experiment involves testing of recombinant protein, it is suggested that you include endogenous (non-transfected) positive controls to validate your results and prove that your

antibodies are working. Detection of recombinant proteins presents a few challenges that should be considered. First, the recombinant protein may fold differently than the native form which could prevent the antibody from accessing its target epitope. This is especially common in cases with tagged proteins. With this in mind, tags should always be placed on either the N or C terminus of the recombinant protein. Secondly, it is important to make sure that the recombinant protein contains the immunogen sequence for your antibody.

Recommended Protocols

Tissue preparation is a key to successful IHC experiments. Since no universal tissue preparation method will be ideal for all sample and tissue types, all protocols given here are intended as a starting point from which the experimenter must optimize as needed. All conditions should be standardized in order to ensure reproducible results. Keep in mind that you must be careful not to allow tissues to dry out at any time.

1. IHC (Paraffin Sections)

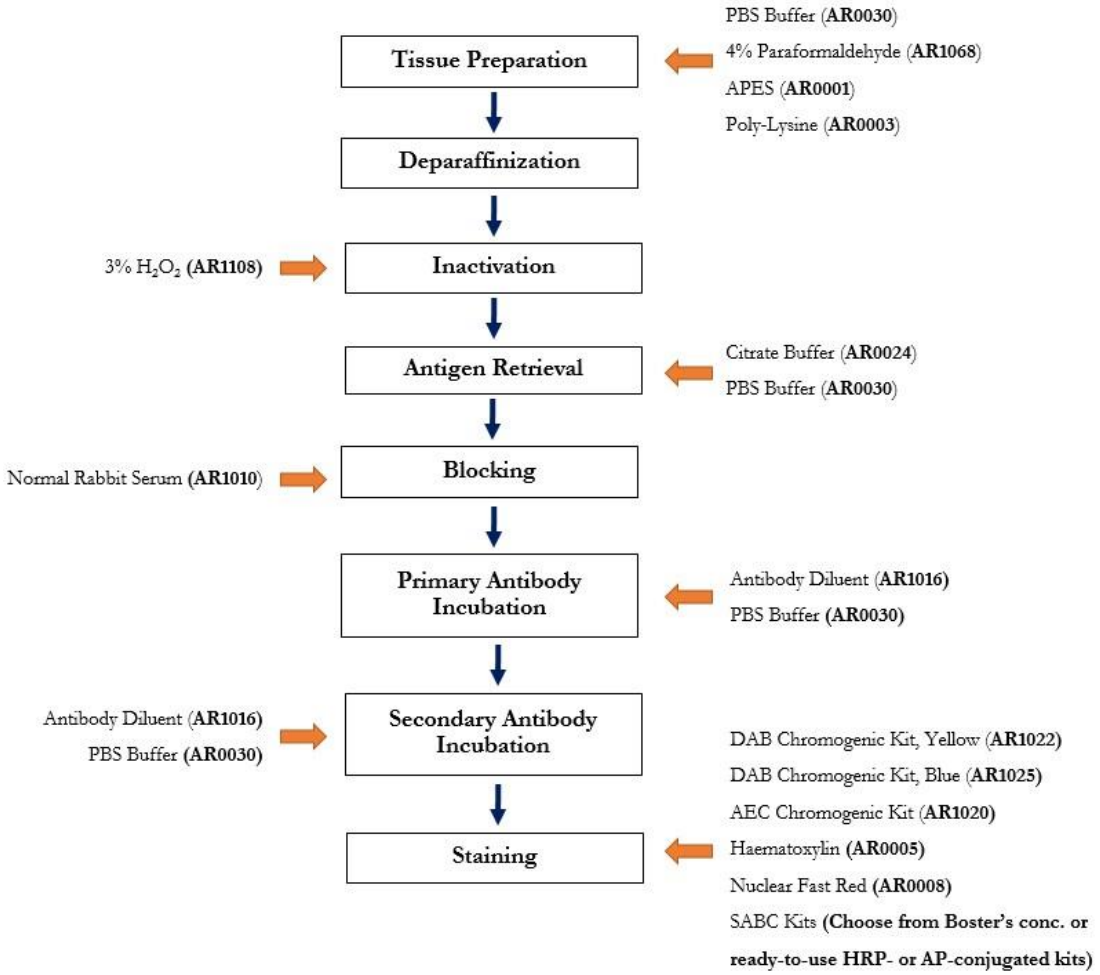

Figure 7. IHC (Paraffin Sections) Workflow with Applicable Boster's Reagents.

(A) Tissue Preparation

(i) Paraformaldehyde Cooling and Dehydration

- Harvest fresh tissue and place it in a dish filled with ice-cold PBS buffer
- Wash the tissue thoroughly with PBS to remove blood (Use forceps to remove connective tissues)
- Cut the tissue into slices of thickness of 3 mm or less

- Immerse the slices in 4% paraformaldehyde at room temperature for 8 min
- Immerse the slices in 4% paraformaldehyde (pre-cool at 4°C) for 6 to 7 hrs. The paraformaldehyde volume should be 20X greater than the tissue volume by weight
- Wash the tissue 3X with PBS (1 min each)
- Dehydrate the tissue by immersing the tissue sequentially as follows:
  - 1X into 80% ethanol (1 hr at 4°C)
  - 1X into 90% ethanol (1 hr at 4°C)
  - 3X into 95% ethanol (1 hr each at 4°C)
  - 3X into 100% ethanol (1 hr each at 4°C)
  - 3X into dimethylbenzene (0.5 hr each at room temperature)

(ii) Liquid Paraffin Section

- Prepare the first portion of liquid paraffin in a suitable bath and allow the paraffin to reach and maintain at 60°C
- Immerse the tissue 2X into the paraffin bath (2 hrs each)
- Prepare the second portion of liquid paraffin in a suitable bath and allow the paraffin to reach and maintain at 60°C
- Pour the second portion of paraffin into a mold
- Quickly transport the tissue from the paraffin bath to the mold with paraffin
- Incubate the tissue at room temperature until it coagulates
- Store the tissue at 4°C

(iii) Section Slicing and Incubation

- Secure the paraffin section on slicer
- Slice one to two pieces of section to adjust the slicer so that the section and blade are parallel
- Slice the remaining section carefully with ~5 μm thick
- Incubate the sliced section in 40 to 50°C water to unfold
- Mount the tissue section onto Poly-Lysine or APES coated glass slides
- Incubate the slides overnight at 37°C

(B) Dewaxing/Deparaffinization

- Prepare the following reagents:
  - 90% dimethylbenzene
  - 95% dimethylbenzene
  - 100% dimethylbenzene
  - 90% ethanol
  - 95% ethanol
  - 100% ethanol
- Sequentially immerse paraffin sections into:
  - 90% dimethylbenzene (for 7 min)
  - 95% dimethylbenzene (for 7 min)
  - 100% dimethylbenzene (for 7 min)
  - 90% ethanol (for 7 min)

- 95% ethanol (for 7 min)
- 100% ethanol (for 7 min)

- Wash the slides with water to remove ethanol

Note: The process of dewaxing should be done in a fume hood at room temperature in summer. When the temperature is lower than 18°C, it is recommended to dewax at 50°C.

(C) Inactivation

- Immerse dewaxed paraffin section into the 3% H_2O_2 at room temperature for 10 min
- Wash the section 3X to 5X with distilled water (total 3 to 5 min)

(D) Antigen Retrieval (Heat Induced Epitope Retrieval: HIER)

- Immerse the paraffin sections in citrate buffer
- Heat the buffer in microwave and turn it off when the buffer has boiled
- Keep the boiled buffer in microwave for 5 to 10 min
- Repeat the heating as outlined above 1X to 2X
- Cool the slides until it reaches room temperature
- Wash the sections 1X to 2X with PBS

(E) Blocking

- Add 5% BSA blocking solution or normal goat serum to the HIER treated samples
- Incubate the samples at 37°C for 30 min
- Discard extra liquid (No washing required)

(F) Primary Antibody Incubation

- Dilute primary antibody with antibody diluent to the concentration recommended by the antibody manufacturer
- Add the diluted antibody to the samples and incubate at 37°C overnight
- Wash the samples 2X with PBS (20 min each)

(G) Secondary Antibody Incubation

- Dilute biotinylated secondary antibody with antibody diluent to the concentration recommended by the antibody manufacturer
- Add the diluted antibody to the samples and incubate at 37°C for 30 min
- Wash the samples 2X with PBS (20 min each)

(H) Staining

- Add Strept-Avidin Biotin Complex (SABC) HRP- or AP-conjugated reagents to the samples
- Incubate the samples at 37°C for 30 min
- Wash the samples 3X with PBS (20 min each)
- Add a suitable amount of DAB reagent to the samples and incubate in dark at room temperature for 10 to 30 min
- Monitor the tissue staining intensity under a bright-field microscope*
- Wash the samples 3X to 5X with distilled water

- Counterstain (if necessary)
  - Add haematoxylin to the sample
  - Dehydrate
  - Immerse the paraffin sections 2X in dimethylbenzene (7 min each)
- Check the tissue staining intensity under a bright-field microscope

* If the staining background is too high, wash the section 4X with 0.01-0.02% TWEEN 20 PBS and 2X with pure PBS after the SABC reaction and before DAB staining. Then use DAB to stain the samples.

2. IHC (Frozen Sections)

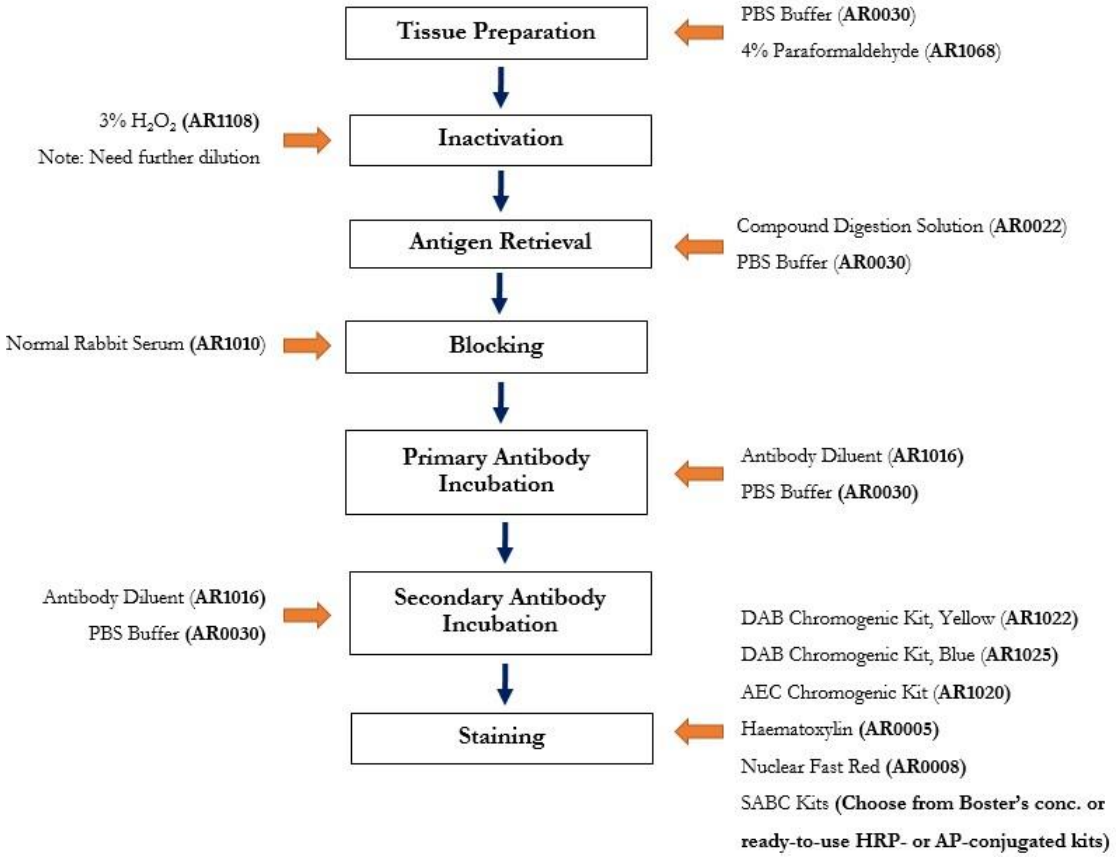

Figure 8. IHC (Frozen Sections) Workflow with Applicable Boster's Reagents.

(A) Tissue Preparation

(i) Snap Freezing and OCT Embedding

- Harvest fresh tissue and place it in a dish filled with ice-cold PBS buffer
- Wash the tissue thoroughly with PBS to remove blood (Use forceps to remove connective tissues)
- Cut the tissue into slices of thickness of 3 mm or less
- Immediately snap freeze the tissue in iso-pentane cooled in dry ice and keep the tissue at -70°C (Do not allow frozen tissue to thaw before cutting)
- Prior to cryostat sectioning, position the tissue in a mold (which can be simply made by using tin foil) and cover the tissue completely in Optimal Cutting Temperature (OCT) embedding medium
- Use forceps to take the bottom part of mold into liquid nitrogen for 1 to 2 min (The OCT should change to white)

(ii) Cryostat Sectioning

- Pre-cool a slicer box and detector to -22°C and -24°C, respectively (Ensure the completeness and smoothness of blade)
- Place the tissue from the mold to the detector where the tissue is fixed

- Quickly and carefully slice the cryostat sections at 5-10 μm and mount them on gelatin-coated histological slides. Note that:
  - Use coverslip to take sliced tissue
  - Cryostat temperature should be between -15°C and -23°C
  - The sections will curl up if the specimen is too cold
  - The sections will stick to the knife if the specimen is too warm
- Air dry the sections at room temperature for 30 min to prevent them from falling off the slides during antibody incubations
- Store the slides at -70°C. Note that:
  - The slides can be stored unfixed for several months at -70°C
  - Frozen tissue samples saved for later analysis should be stored intact
- Immediately add 50 μL of ice-cold fixation buffer to each tissue section upon removal from the freezer
- Fix frozen section by immersing it into 4% paraformaldehyde at 2-8°C for 8 min (Or optimally at -20°C for 20 min)
- Wash the section 3X with PBS and allow it to dry at room temperature for 30 min

(B) Inactivation

- Mix H_2O_2 with distilled water (v/v: 1:50)
- Immerse frozen section or cell climbing slice into the diluted H_2O_2 at room temperature for 10 min
- Wash the section 3X distilled water (1 min each)

(C) Antigen Retrieval (Proteolytic Induced Epitope Retrieval: PIER)

- Dry the frozen sections with filter paper
- Add compound digestion solution (e.g. Trypsin solution or other enzymatic antigen retrieval solution) to the sections or slices
- Incubate the sections at room temperature for 3 to 5 min
- Wash the sections with 3X PBS (5 min each)

(D) Blocking

- Add 5% BSA blocking solution or normal goat serum to the PIER treated samples
- Incubate the samples at 37°C for 30 min
- Discard extra liquid (No washing required)

(E) Primary Antibody Incubation

- Dilute primary antibody with antibody diluent to the concentration recommended by the antibody manufacturer
- Add the diluted antibody to the samples and incubate at 37°C overnight
- Wash the samples 2X with PBS (20 min each)

(F) Secondary Antibody Incubation

- Dilute biotinylated secondary antibody with antibody diluent o the concentration recommended by the antibody manufacturer

- Add the diluted antibody to the samples and incubate at 37°C for 30 min
- Wash the samples 2X with PBS (20 min each)

(G) Staining

- Add Strept-Avidin Biotin Complex (SABC) HRP- or AP-conjugated reagents to the samples
- Incubate the samples at 37°C for 30 min
- Wash the samples 3X with PBS (20 min each)
- Add a suitable amount of DAB reagent to the samples and incubate in dark at room temperature for 10 to 30 min
- Monitor the tissue staining intensity under a bright-field microscope*
- Wash the samples 3X to 5X with distilled water
- Counterstain (if necessary)
  - Add haematoxylin to the sample
  - Dehydrate
  - Immerse the paraffin sections 2X in dimethylbenzene (7 min each)
- Check the tissue staining intensity under a bright-field microscope

* If the staining background is too high, wash the section 4X with 0.01-0.02% TWEEN 20 PBS and 2X with pure PBS after the SABC reaction and before DAB staining. Then use DAB to stain the samples.

3. ICC/IF (Cell Climbing Slices)

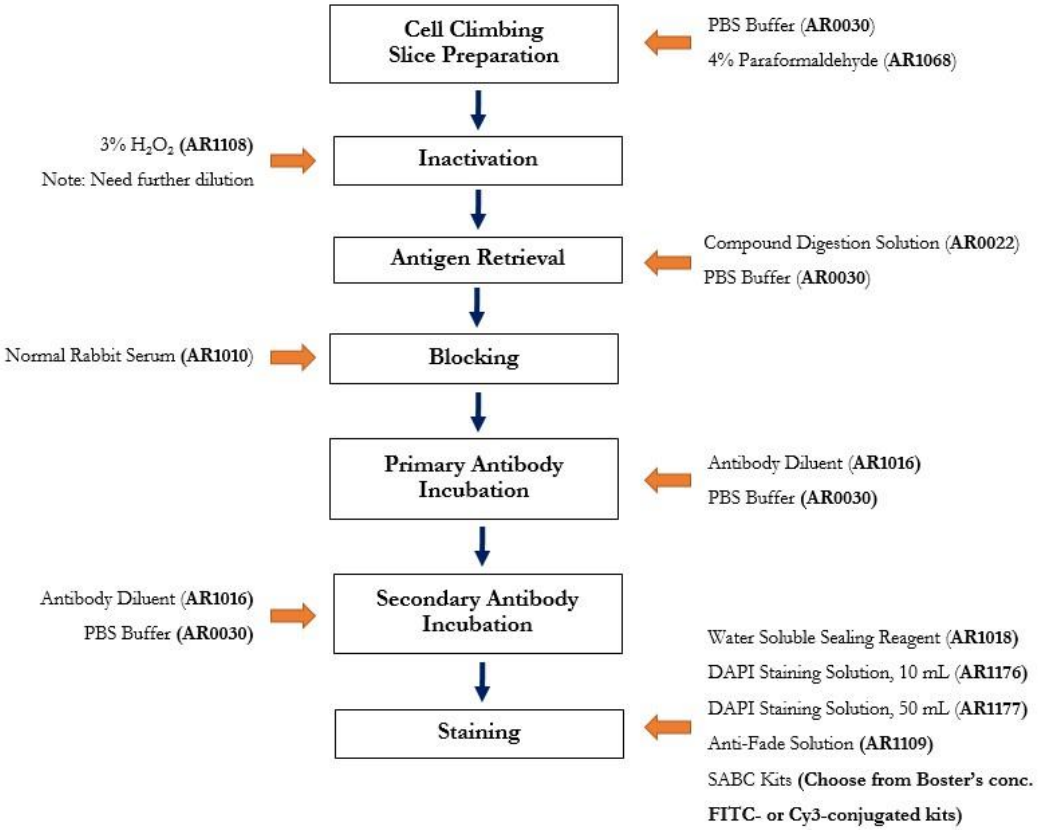

Figure 9. ICC/IF Workflow with Applicable Boster's Reagents.

(A) Cell Climbing Slice Preparation

- Place settled coverslip in culture bottle or perforated plate
- Take out coverslip after cell growth has reached 60%
- Wash the coverslip 3X with PBS to remove culture medium
- Immerse the coverslip (cells face up) into cold acetone or 4% paraformaldehyde or neutral formalin for 10 to 20 min (Close the lid to prevent evaporation)
- Wash the coverslip 3X with PBS
- Put the coverslip on filter paper (cells face up)
- Remove the liquid on the coverslip and allow it to dry for 8-10 hrs
- To thaw the slice, wash with neutral PBS at room temperature for 10-15 min (The cell climbing slice can be stored in gelatin at -20°C for one week.)

Note: This fixation procedure using paraformaldehyde and formalin fixatives may cause autofluorescence in the green spectrum. In this case, you may try fluorophores in the (i) red range or (ii) infrared range if you have an infrared detection system.

(B) Inactivation

- Mix H_2O_2 with distilled water (v/v: 1:50)
- Immerse frozen section or cell climbing slice into the diluted H_2O_2 at room temperature for 10 min

- Wash the section 3X distilled water (1 min each)

(C) Antigen Retrieval (Proteolytic Induced Epitope Retrieval: PIER)

- Dry the cell slices with filter paper
- Add compound digestion solution (e.g. Trypsin solution or other enzymatic antigen retrieval solution) to the slices (We recommend the addition of 0.1% Triton to the samples before the digestion. This reduces surface tension and allows reagents to easily cover the entire sample.)
- Incubate the slices at room temperature for 10 min
- Wash with 3X PBS (10 min each)

(D) Blocking

- Add 5% BSA blocking solution or normal goat serum to the PIER treated samples
- Incubate the samples at 37°C for 30 min
- Shake off extra liquid and dry the samples with filter paper (No washing required)

(E) Primary Antibody Incubation

- Dilute primary antibody with antibody diluent to the concentration recommended by the antibody manufacturer
- Add the diluted antibody (Recommended concentration: 0.4 μg to 2 μg) to the samples and incubate at 4°C overnight
- Wash the samples 3X with PBS (15 min each)

(F) Secondary Antibody Incubation

- Dilute biotinylated secondary antibody with antibody diluent to the concentration recommended by the antibody manufacturer
- Add the diluted antibody to the samples and incubate at 37°C for 30 min
- Wash the samples 3X with PBS (8 min each)

(G) Staining

- Add Strept-Avidin Biotin Complex – Fluorescence Iso-Thio-Cyanate (SABC-FITC) or Strept-Avidin Biotin Complex – Cyanine-3 (SABC-Cy3) reagents to the samples
- Incubate the samples at 37°C for 30 min (Avoid light)
- Wash the samples 2X with PBS (Total 2 hrs)
- Seal the slices with water soluble sealing reagent
- Monitor the staining intensity under a fluorescence microscope
- Counterstain by adding DAPI staining solution to the sample
- Check again the staining intensity under a fluorescence microscope
- For slide storage without significant decay in fluorescence signal, add 20 μL of anti-fade solution to the sample followed by a cover glass (Avoid bubbles)

Troubleshooting Guide

The following guide serves as a checklist for the possible causes and solutions with respect to some of the most commonly encountered problems from the IHC assays.

1. No or Weak Staining

	Possible Cause	Solution
1	Slides lose signal over time during storage	Prepare slides with freshly-slice tissues Store slides at 4°C Do not bake slides before storage
2	The antibody used is not suitable for IHC procedures which detect proteins in its native conformation	Check the antibody datasheet to make certain that it has been validated for IHC applications Check the antibody is applicable to the right IHC samples (paraffin sections vs. frozen samples) Perform Western blot in both its native and denatured forms to ensure that the antibody detects the native form
3	Fixation procedures (using formalin and paraformaldehyde fixatives) have masked the epitope that the antibody recognizes	Use different antigen retrieval methods to unmask the epitope (HIER or PIER) Fix the sections in a shorter time
4	The primary and/or secondary antibody have lost its activity due to improper storage, dilution or excessive freezing and thawing	Run positive controls to ensure that the primary and/or secondary antibody is working properly Store the antibodies according to manufacturer instructions Avoid contamination and light on antibodies
5	Insufficient deparaffinization	Increase the deparaffinization time Use fresh dimethylbenzene
6	The protein is located in the nucleus and the antibody cannot penetrate the nucleus	Add a permeabilizing agent (e.g. Triton X) to the blocking buffer and antibody dilution

		buffer
7	The PBS buffer has contaminated with bacteria that damage the phosphate groups on the protein of interest	Add 0.01% azide in the PBS antibody storage buffer Use fresh sterile PBS
8	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 the primary antibody was raised in mouse, an anti-mouse secondary antibody should be used) Check that the isotypes of the primary and secondary are compatible
9	The protein is not present in the tissue of interest or has not sufficiently expressed	Run positive controls to ensure that target protein is present in the tissue Include an amplification step in your protocol Use higher antibody concentration
10	Insufficient antibody to detect protein of interest	Use a higher antibody concentration Incubate for a longer time (e.g. overnight) at 4°C
11	Tissues dry out	Cover the tissues in liquid at all time during the experiment

2. High Background

	Possible Cause	**Solution**
1	The blocking serum is incorrect	Make sure to block according to the provided protocol
2	Blocking is insufficient (**Do not over-block the tissue** because antigenic sites may be masked)	Increase blocking incubation period Change blocking reagent: (a) For sections: 10% normal serum (1 hr) (b) For cell cultures: 1-5% BSA (0.5 hr)
3	The primary antibody concentration is too high	Titrate the antibody to determine the optimal concentration

		Incubate at 4°C
4	Non-specific binding by secondary antibody	Run a secondary control without primary antibody: If you see staining with your secondary only: (a) Change your secondary antibody or (b) Use secondary antibody that has been pre-adsorbed against the immunoglobulin of the species from which your samples were obtained Block your sample with serum from the same species as the host in which the secondary antibody was raised
5	Endogenous peroxide or phosphatase is active	Quench the endogenous peroxidase or phosphatase activity by enzyme inhibitors: (a) Peroxidase: H_2O_2 and methanol (v/v: 0.3%:99.7%) (b) Phosphatase: 2 mM Levamisol
6	Too much amplification (Refer to solution #9 from no/weak staining)	Reduce amplification incubation time Dilute the secondary antibody
7	Too much substrate was applied (enzymatic detection)	Further dilute substrate Reduce substrate incubation time Choose substrate of higher S/N ratio e.g. Metal-enhanced DAB
8	Tissue section is not thin enough for reagent penetration	Prepare thinner section
9	Incubation temperature is too high	Incubate samples at 4°C
10	Primary antibody was raised in the same species as source of tissue (therefore, secondary antibody recognizes and binds to everywhere on the entire tissue because it was raised against that species)	Use primary antibody raised against a species which is different from the source of tissue Use biotinylated primary antibody and conjugated streptavidin for the detection system
11	Secondary antibody binds endogenous IgG	Include control slide stained without the

		primary antibody to confirm whether the secondary antibody is the source of the background
12	Fixation reagents are still present (Due to insufficient tissue washing)	Wash the tissues extensively with PBS buffer
13	Reaction between chromogens and PBS buffer in tissue or cell samples	Before incubating with the substrate, use Tris buffer to wash the samples
14	Membrane damage by permeabilization	Use a less stringent detergent such as Tween 20 (instead of Triton X) Remove permeabilizing agent from your buffers
15	Insufficient deparaffinization	Increase the deparaffinization time Use fresh dimethylbenzene
16	High levels of endogenous biotin in biotin-based detection systems for samples (e.g. liver and kidney tissues)	Perform biotin block after normal blocking procedure (before primary antibody incubation) Use polymer-based detection
17	Use of polyclonal primary antibody	Use monoclonal primary antibody to reduce cross-reactivity

FAQs

1. What is immunohistochemistry?

Immunohistochemistry (IHC) is the identification and localization of antigens or proteins in tissue preparations through the use of antibodies labeled with enzymes or fluorescent dyes. The antibody-antigen complexes are generally visualized after a reaction step with a substrate that produces a calorimetric result or with specialized imaging equipment to detect fluorescence.

2. How do you determine the starting IHC antibody concentrations?

Optimal antibody concentration will give you good staining with minimum background. It is generally a good idea to conduct a titration experiment with new antibodies using a range of concentrations that includes the manufacturer's suggested dilution. For example, if the manufacturer suggests a dilution of 1:100, you might also test 1:50, 1:200, 1:500 and 1:1000. This should help you determine the ideal dilution for your conditions.

For antibodies with no suggested dilution, it is advisable to begin with titration experiments in the following ranges based on the antibody format:

- Tissue culture supernatant: neat to 1/10
- Ascites: 1/100
- Whole anti-serum: 1/50 to 1/100
- Purified antibody: 5 μg/mL

3. What is the best way to mount slides?

Tissue sections should be mounted on positively charged or APES (Amino-Propyl-Triethoxy-Silane) coated slides. After mounting the sections, the slides should be left at room temperature overnight to allow water that may be trapped under the section to dry out. In the event that tissue sections adhere to the slide, it may be incubated at 60ºC for a few hours.

4. How should IHC slides be stored?

Slides with paraffin-embedded tissue sections can be kept for up to 3 years at 2-8°C, depending on the antigen in question. Frozen tissue sections from snap-frozen tissue blocks should be carefully wrapped in aluminum foil and stored at -20°C or lower for up to six months.

5. What is the stability of IHC slides after staining?

This depends on your fixation and mounting protocols as well as the type of stain used. For example, slides stained using chromogenic methods are stable for years, whereas those using immunofluorescent methods are stable on the order of months after staining if held at 4°C and protected from light. The longevity of fluorescent stains can be improved by using a fluorescence-protective medium and sealing the preparation off from air with nail polish.

Additionally, adding an antioxidant to the mounting medium can prevent photo-bleaching due to reactive oxygen species (ROS).

6. Should I use frozen or paraffin sections?

Sample preparation goes hand-in-hand with the fixation method which, in turn, is influenced by the detection technique (i.e., fluorescence vs. chromogenic). Generally, tissue that has been immersion-fixed in formaldehyde must be paraffin-embedded and then cut by a microtome. However, formaldehyde fixation is not appropriate with phosphorylation-dependent epitopes which have been shown to translocate from the membrane to the cytoplasm following this type of fixation. In these cases, tissue may need to be snap frozen and sectioned with a cryostat.

7. Should I use IHC or IF?

When deciding between immunohistochemistry (IHC) and immunofluorescence (IF), these considerations should be taken into account:

- IHC is used if a fluorescence microscope is not available.
- If co-localization studies are being conducted, IF is a better option because fluorescence can be imaged using a confocal microscope which allows superior localization of labeled proteins within the cellular/tissue setting.
- The signal amplification needed for the experiment: IHC is usually much more sensitive than IF.
- Simplicity: IHC requires an additional step in which the enzyme is reacted with the substrate while IF does not.
- Stability: IHC stains can be preserved for years, if not indefinitely, whereas IF slides are stable only for a period of 1-6 months, depending on the fluorophore used.

8. How does IHC sensitivity compare to IF?

IHC allows superior sensitivity due to two major factors:

- Excellent signal to noise ratio for high density antigens (> 10,000 molecules per cell) can be achieved with a simple indirect IHC. An equivalent IF immunostaining is less sensitive since none of the fluorophore for which filters are commonly available can compare (with the possible exception of Cy3). Other fluorophores (e.g. Pe, Cy5 or APC) either fade rapidly (Pe) or require special filters that are usually not available on standard epifluorescence microscopes.
- For low density antigens (2,000 < x <10,000 molecules per cell), the only reliable and widely available signal amplification methods are with IHC.

9. Should I use Alkaline Phosphatase (AP) or Horseradish Peroxidase (HRP)? How do their sensitivities compare?

HRP and AP are enzyme probes used to detect target proteins through chromogenic, chemiluminescent or fluorescent outputs. HRP produces abundant reaction products in a short

amount of time at physiological pH (7.6) and has higher specific enzyme activity as well as immunological reactivity than AP. However, HRP is associated with nonspecific staining resulting from endogenous peroxide activity in some tissues which can be addressed with peroxidase inhibitors.

Since paraffin inhibits endogenous peroxidase activity, HRP is also the method of choice for paraffin-embedded samples. HRP can be degraded not only by microorganisms but also antibacterial agents used against them. HRP should not be used with reagents containing sodium azide.

AP, on the other hand, has an optimal pH range of 9.0 - 9.6. AP reaction rates are linear so if the sensitivity is insufficient, the reaction can be allowed to proceed for a longer time period. Another advantage for using AP is that it is not inhibited by microorganisms or antibacterial agents such as sodium azide or thimerosal.

While NBT/BCIP substrate for AP is the most sensitive, it is not widely used because the reaction is slow and does not allow adequate nuclear counterstain plus it can produce a diffuse signal. Further, it is incompatible with permanent mounting media. Development using DAB substrate for HRP is far more common because the reaction is fast, deposition is precise, and results are better in color contrast with nuclear stains.

10. Do I need detergent for cell permeation? Ionic vs non-ionic?

Detergents are a class of molecules with unique biochemical properties that enable the disruption of hydrophobic-hydrophilic interactions between molecules in biological samples. If your target antigen has an intracellular location, you will need to use a detergent to allow the antibody access to intracellular compartments.

Ionic detergents consist of a hydrophobic chain and a charged headgroup (either anionic or cationic). Because ionic detergents, such as SDS (anionic), disrupt protein-protein interactions, they are not recommended for studies that involve functioning proteins or protein-protein interactions.

Nonionic detergents consist of uncharged and hydrophilic headgroups. While they disrupt protein-lipid and lipid-lipid associations (cellular membranes), they are mild surfactants because they do not break protein-protein interactions. This allows proteins to be solubilized and isolated in their native (active) form, retaining the protein interactions.

Unfortunately, there is no ideal detergent for all applications as the results may also vary with the conditions and buffers used. Generally, moderate concentrations of mild (nonionic, i.e., Triton X and Tween) detergents are sufficient to permeabilize cell membranes and facilitate the extraction of soluble/native proteins. Using certain buffer conditions, various detergents effectively penetrate between the membrane bilayers at concentrations sufficient to form mixed micelles with isolated phospholipids and membrane proteins. It is best to try out with several detergents and even mix some of the detergents to determine the optimal agent for your experiment.

11. How long should I incubate the primary antibody?

Incubation for an insufficient time period will not produce adequate signal. However, incubations that are too lengthy can result in non-specific staining. The incubation time will vary for each antibody and must be individually determined. Generally, antibodies with known high affinity (i.e., monoclonal) should be used at high dilutions with overnight incubations. Since polyclonal antibodies have varying affinities, one must iteratively determine its optimal incubation.

12. What is the stability for diluted antibody?

Carefully read the manufacturer's instructions and ensure that you store your antibodies properly and protect them from contamination. Optimal storage conditions are unique to each antibody but some general guidelines can be followed. Antibodies must be stored at an appropriate temperature and pH range and frequently in the presence of concentrated (~ 1M) substances such as glycerol or sucrose in order to retain activity and prevent aggregation. Antimicrobial agents are sometimes used to prevent contamination, though this is inappropriate for assays in which HRP is used.

When antibodies are stored in aqueous form at 4°C, a typical shelf life is one month. With this in mind, it is a good idea to aliquot the antibody before diluting and keep the aliquots at -20°C (see exception below) in which case the aliquots are stable for years.

Enzyme-conjugated antibodies should generally be kept at 4°C and never be frozen. Conjugated antibodies, whether conjugated to fluorochromes, enzymes, or biotin, should be stored in dark vials or wrapped in foil because exposure to light can compromise the activity of conjugates. Fluorescent conjugates in particular are susceptible to photo-bleaching and should be protected from light during all phases of an experiment.

13. Should I freeze my antibodies?

Antibodies are vulnerable to some degrees of freezing conditions most likely because of the damage incurred during ice crystal formation which causes antibody to lose its biological activity. With this in mind, freeze-thaw cycles should be avoided. Aliquoting the antibodies and keeping them at -20°C resolve the freeze-thaw issue.

Again, enzyme-conjugated antibodies should generally not be frozen. Instead, they should be kept at 4°C, protected from light and contamination.

14. How should samples be prepared for IHC?

Tissue and cell samples must be harvested and prepared carefully for each IHC study. In order for the incubation steps to work properly, whole tissues must be cut into ultra-thin (5-10 μm) slices or cut into smaller pieces for whole mount IHC. Sample preparation is also closely linked to the method of fixation. Careful consideration must be given to the requirements of the detection technique that will be used (fluorescence vs. chromogenic).

For example, tissue that has been immersion-fixed in formaldehyde must be paraffin-embedded and cut using a microtome. However, some tissues and epitopes cannot be fixed with formaldehyde and therefore must be snap frozen, then sectioned with a cryostat and fixed with alcohol.

15. How should I design the IHC controls?

Appropriate controls are critical to validate the IHC experiment and enable accurate interpretation of results. A well-designed IHC experiment shows that: 1) the antigen is localized to the correct specialized tissues, cell types or subcellular location; 2) the optimization of experimental conditions (i.e., fixation, blocking, antibody incubation, and antigen retrieval steps) generates a robust and specific signal. Positive and negative controls must be included to validate staining and identify or rule out experimental artifacts. In addition, variations in antibody specificity, experimental conditions, biological conditions between tissue types should be carefully considered.

Ordering Information

Boster currently offers a variety of Strept-Avidin Biotin Complex (SABC) 1) HRP-, 2) AP-, 3) fluorescent dye- and 4) double-label- conjugated reagents for IHC and ICC/IF applications.

1) SABC HRP-Conjugated Kits

Host/Isotype	Format	No. Stained Slides per Kit	Cat. No.
Mouse IgG	Concentrated	1500 - 1800	SA2001
Rabbit IgG	Concentrated	1500 – 1800	SA2002
Goat IgG	Concentrated	1500 – 1800	SA2003
Human IgG	Concentrated	1500 – 1800	SA2004
Rat IgG	Concentrated	1500 – 1800	SA2005
Mouse/Rabbit IgG	Concentrated	1500 – 1800	SA2010
Mouse/Rabbit IgG	Ready-to-Use	150 – 180	SA1020
Mouse IgG	Ready-to-Use	150 – 180	SA1021
Rabbit IgG	Ready-to-Use	150 – 180	SA1022
Goat IgG	Ready-to-Use	150 – 180	SA1023
Human IgG	Ready-to-Use	150 – 180	SA1024
Rat IgG	Ready-to-Use	150 – 180	SA1025
Mouse IgM	Ready-to-Use	150 – 180	SA1026
Mouse IgG*	Ready-to-Use	150 – 180	SA1027
Rabbit IgG*	Ready-to-Use	150 – 180	SA1028

* Specially designed for IHC with frozen sections that exhibit low background

2) SABC AP-Conjugated Kits

Host/Isotype	Format	No. Stained Slides per Kit	Cat. No.
Mouse/Rabbit IgG	Ready-to-Use	150 – 180	SA1050
Mouse IgG	Ready-to-Use	150 – 180	SA1051
Rabbit IgG	Ready-to-Use	150 – 180	SA1052
Goat IgG	Ready-to-Use	150 – 180	SA1053
Human IgG	Ready-to-Use	150 – 180	SA1054
Rat IgG	Ready-to-Use	150 – 180	SA1055
Mouse IgM	Ready-to-Use	150 – 180	SA1056

3) SABC FITC-Conjugated Kits

Host/Isotype	Format	No. Stained Slides per Kit	Cat. No.
Mouse IgG	Concentrated	1500 - 1800	SA1062
Mouse IgM	Concentrated	1500 – 1800	SA1063
Rabbit IgG	Concentrated	1500 – 1800	SA1064
Goat IgG	Concentrated	1500 – 1800	SA1066
Human IgG	Concentrated	1500 – 1800	SA1068
Rat IgG	Concentrated	1500 – 1800	SA1069

4) SABC Cy3-Conjugated Kits

Host/Isotype	Format	No. Stained Slides per Kit	Cat. No.
Mouse IgG	Concentrated	1500 - 1800	SA1072
Mouse IgM	Concentrated	1500 – 1800	SA1073
Rabbit IgG	Concentrated	1500 – 1800	SA1074
Goat IgG	Concentrated	1500 – 1800	SA1076
Human IgG	Concentrated	1500 – 1800	SA1078
Rat IgG	Concentrated	1500 – 1800	SA1079

5) SABC Double-Labeled (FITC and HRP) Conjugated Kits

Host/Isotype	Format	No. Stained Slides per Kit	Cat. No.
Mouse IgG	Concentrated	1500 - 1800	SA1080
Mouse IgM	Concentrated	1500 – 1800	SA1081
Rabbit IgG	Concentrated	1500 – 1800	SA1082
Goat IgG	Concentrated	1500 – 1800	SA1083
Human IgG	Concentrated	1500 – 1800	SA1084
Rat IgG	Concentrated	1500 – 1800	SA1085

Super Vision (SV) series is one of the innovation designs from Boster. This polymer-based IHC detection system is designed to both enhance the sensitivity and reduce the background stain while providing a two-step alternative to traditional IHC detection methods. Using a polymeric labeling method, HRP is linked to the secondary antibodies to form a huge octopus-like molecule antibody-enzyme complex in the SV system. The HRP-conjugated polymer enables signal amplification and permeation in tissues and cells. The system takes advantages of three properties of the HRP-conjugated secondary antibody polymer:

- The quantity of HRP conjugated to the secondary antibody IgG is much larger than that conjugated to the other polymers, and thus providing extremely high sensitivity.
- The HRP-conjugated secondary antibody polymer is capable of penetrating tissues and cells due to its small molecular weight and absence of inert polymer skeleton.
- Without the use of avidin, the macro-molecule polymer avoids the interference from endogenous biotin, reducing false positive rate and background staining.

SV Detection Kits

Host/Isotype	Size	Format	No. Stained Slides per Kit	Cat. No.
Mouse IgG	10 mL	Ready-to-Use	120 - 150	SV0001-1
Mouse IgG	100 mL	Ready-to-Use	1200 – 1500	SV0001-2
Rabbit IgG	10 mL	Ready-to-Use	120 - 150	SV0002-1
Rabbit IgG	100 mL	Ready-to-Use	1200 – 1500	SV0002-2
Goat IgG	10 mL	Ready-to-Use	120 - 150	SV0003-1
Goat IgG	100 mL	Ready-to-Use	1200 – 1500	SV0003-2

We also offer a variety of IHC and ICC/IF ancillary reagents:

Product	Size	Cat. No.
3% H_2O_2 Solution	50 mL	AR1108
4% Paraformaldehyde	500 mL	AR1068
AEC Chromogenic Kit	1 Kit	AR1020
Amino Propyl Tri-Ethoxy Silane (APES)	10 mL	AR0001
Antibody Diluent Solution	100 mL	AR1016
Anti-Fade Solution	10 mL	AR1109
Anti-Peeling Slide	50 Pieces	AR1065
Buffer (PBS)	2 L	AR0030
Buffer (TBS)	2 L	AR0031
Citrate Buffer	2 L	AR0024
Compound Digestion Solution	50 mL	AR0022
DAB Chromogenic Kit (Yellow)	1 Kit	AR1022
DAB Chromogenic Kit (Blue)	1 Kit	AR1025
DAPI Staining Solution	10 mL	AR1176
DAPI Staining Solution	50 mL	AR1177
Haematoxylin	12 mL	AR0005
Normal Rabbit Serum (Blocking)	100 mL	AR1010
Nuclear Fast Red	12 mL	AR0008
Poly-Lysine	10 mL	AR0003
Water Soluble Sealing Reagent	50 mL	AR1018

We take pride in our 2100+ antibodies that have been validated for IHC applications. Our **Picoband™** antibodies (Catalog numbers started with "PB") are carefully developed with the best immunogens to ensure minimal cross reactivity and maximum affinity. See the **Antibody Gene List** for a complete list of genes that we provide associated antibodies.

Antibody Gene Index

AAMP
ABCB1
ABCB11
ABCB4
ABCB6
ABCC1
ABCC4
ABCD3
ABCG2
ABI1
ABL2
ABR
ACE
ACTA1
ACTA2
ACTB
ACTG1
ACTN2
ACTN4
ADA
ADAM19
ADAMTS2
ADAMTS5
ADAR
ADIPOQ
AFP
AGER
AGTR1
AHR
AHSG
AIFM1
AIM2
AIMP2
AKT2
AKT3
ALDH1A1
ALDH2
ALDH3A1
ALDH3A2
ALK
ALOX12
ALOX15
ALOX5
ALPL
AMD1
AMY1A
ANGPT1
ANXA1
ANXA10
ANXA2
ANXA3
ANXA4
ANXA5
ANXA6
ANXA7
APAF1
APBB1
APBB1IP
APCS
APEX1
APEX2
API5
APLP1
APOA1
APOBEC3G
APOE
APP
APPL1
AQP1
AQP2
AQP3
AQP4
AQP5
AQP6
AR
ARG2
ARID1A
ARRB1
ARRB2
ARSA
ASPH
ATF1
ATF2
ATG14
ATP2A1
ATP2A2
ATP2A3
ATP5H
ATP7B
ATXN1
ATXN2
ATXN3
B3GNT8
BAG1
BAG2
BAG3
BAG5
BAK1
BCAR3
BCAT1
BCAT2
BCL2
BCL2L1
BCL2L2
BCL3
BDNF
BECN1
BGLAP
BID
BIK
BIRC5
BIRC7
BMP2
BMP5
BMPR1B
BRCA1
BTK
BUB3
C1QBP
C5
CA1
CA2
CA3
CACYBP
CALB1
CALB2
CALCA
CALD1
CALM1
CALM2
CAMKK1
CAMKK2
CAPN1
CASP1
CASP10
CASP12
CASP14
CASP3
CASP4
CASP6
CASP7
CASP8
CASP9
CASR
CAT
CAV1
CAV2
CBS
CCL26
CCNA2
CCND1
CCNT1
CCR1
CCR3
CCR4
CCR5
CCT2
CCT4
CCT5
CCT8
CD14
CD163
CD19
CD1D
CD2
CD22
CD244
CD27
CD33
CD34
CD36
CD3E
CD40LG
CD44
CD46
CD47
CD55
CD58
CD59
CD63
CD68
CD79A
CD79B
CD82
CD8A
CD9
CDC25A
CDC25B
CDC34
CDC42
CDC6
CDCP1
CDH1
CDH2
CDH3
CDH5
CDK1
CDK2
CDK4
CDK9
CDKN2D
CEACAM1
CEACAM5
CEBPA
CEBPB
CFB
CFL1
CFL2
CFTR
CGB
CHEK2
CHGA
CHRM1
CHRM2
CHUK
CIITA
CISH
CLDN2
CLOCK
CLU
CMA1
CNN1
CNN2
CNTF
COL1A1
COL4A1
COL4A2
COL4A3
COL4A4
COL4A5
COL4A6
COMT
COPS5
CP
CPB2
CPT1B
CRK
CRY2
CRYAA
CSF1
CSF2
CSK
CSNK1A1
CST3
CSTA
CSTB
CTBP1
CTBP2
CTBP2
CTCF
CTGF
CTH
CTNNA1
CTNNB1
CTSD
CTSK
CUEDC2
Cul1
CUL4B
CX3CL1
CXCL10
CXCL13
CXCL16
CXCL9
CXCR1
CXCR3
CXCR4
CXCR6
CYBB
CYCS
CYP11A1
CYP19A1
CYP1A1
CYP1A2
CYP1B1
CYP24A1
CYP27B1
CYP2D6
CYP2E1
CYP2U1
CYP7A1
DAXX
DCC
DCN
DDAH2
DDB1
DDT
DDX4
DDX5
DEFB1
DES
DHFR
DIABLO
DICER1
DISC1
DKK3
DLD
DLG1
DLG4
DMD
DMRT1
DNAJB1
DRD1
DRD2
DRD3
DSG2
DUT
EBAG9
ECI1
EDNRB
EGF
EGR1
EIF2S1
EIF4A2
EIF6
ELAVL4
ELN
EME1
ENG
ENO2
EP300
EPAS1
EPHA1
EPHA2
EPHB1
EPHB3
EPO
ESR1
EWSR1
F2R
FABP1
FABP2
FABP3
FABP4
FABP5
FABP6
FAP
FAS
FASLG
FASN
FBXL4
FCER2
FCGRT
FER
FGF1
FGF19
FGF2
FGF7
FGF8
FGF9
FGFR1
FGFR2
FGFR3
FGFR4
FHIT
FIGF
FLG
FLI1
FLOT2
FMO4
FN1
FOSB
FOXO3
FOXP1

Antibody Gene Index (Continued)

FSHB
FUT1
FXYD1
FXYD5
FZD1
GABRA1
GAD2
GAP43
GAPDH
GCG
GCLC
GDNF
GFAP
GFRA1
GH1
GIP
GJA1
GJA3
GJA4
GJA5
GJB1
GLI1
GLRX2
GNAQ
GORAB
GPX1
GPX2
GPX4
GRB10
GRB7
GRIA1
GRIA2
GRIN1
GRIN2A
GRK5
GSN
GSTP1
GZMA
HAVCR1
HCRTR1
HDAC11
HDAC2
HDAC3
HDAC6
HDAC8
HDC
HEXA
HEXB
HIF1A
HINT1
HLA-A
HLA-C
HLA-DMB
HMGB2
HMGB3
HMMR
HMOX1
HMOX2
HNF1B
HNF4A
HOXA3
HOXA4
HP
HRG
HSD11B1
HSD11B2
HSD17B1
HSD17B10
HSD17B2
HSD17B4
HSD17B6
HSF1
HSF2
HSP90AA1
HSP90AB1
HSP90B1
HSPA1A
HSPA1B
HSPA2
HSPA5
HSPA8
HSPA9
HSPB1
HSPB2
HSPD1
HSPE1
HSPG2
HSPH1
HTR1A
HTR2A
HTR3A
HYAL1
HYAL3
IBSP
ICAM1
IDH1
IDH2
IDO1
IDS
IFITM1
IFNG
IGF1
IGF1R
IGF2R
IGFBP3
IKBKB
IKZF1
IL10
IL16
IL18
IL1A
IL2RA
IL3
IL4
IL4R
IL7
IL8
INA
INPPL1
INS
INSL3
IRAK2
IRAK3
IRF1
IRF4
IRF5
IRS1
ISG15
ITGA1
ITGA2B
ITGA3
ITGA4
ITGA6
ITGAE
ITGAM
ITGAV
ITGB1
ITGB2
ITGB3
ITGB4
ITLN1
ITPR1
ITPR3
IVL
JAK2
JUNB
JUP
KCNA4
KCNB1
KCNIP2
KCNMA1
KDR
KIF2C
KIN
KIT
KITLG
KLK1
KLK11
KLK2
KLK5
KLK6
KNG1
KRT1
KRT18
KRT19
KRT7
KRT8
LALBA
LAMA1
LAMA2
LAMB1
LAMB2
LAMC1
LAMC2
LAMC3
LASP1
LAT
LCK
LCN1
LCN2
LEPR
LGALS1
LGALS3
LHCGR
LIF
LMNA
LMNB1
LMNB2
LOXL1
LPXN
LTBR
LUM
LYZ
MAD1L1
MADCAM1
MAG
MAOA
MAOB
MAP1A
MAP2
MAP2K1
MAP2K3
MAP2K7
MAP3K1
MAP3K3
MAP3K8
MAPK1
MAPK3
MAPK6
MAPK8
MAPK13
MAPT
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MBP
MCAM
MCL1
MCM2
MCM3
MCM5
MCM6
MCM7
MDM4
MEF2A
MEF2C
MFGE8
MGMT
MGP
MIF
MKI67
MME
MMP14
MMP16
MMP2
MMP7
MMP8
MMP9
MNAT1
MPO
MRGPRX1
MS4A1
MSH2
MSLN
MTA1
MT-CO1
MTDH
MUC1
MUC2
MUC5AC
MYBL2
MYC
MYD88
MYH1
MYH2
MYH11
MYH7
NAMPT
NBN
NCAM1
NDRG1
NDRG2
NDUFA1
NEFH
NEFL
NES
NFATC2
NFE2L2
NFKB1
NFKB2
NFKBIA
NFKBIB
NLRP3
Nlrp4g
NME1
NME2
NMI
NOD1
NONO
NOS1
NOS2
NOS3
NOX1
NOX4
NOX5
NPHS1
NPHS2
NPM1
NPY
NR3C1
NRF1
NRG1
NRP1
NTF3
NTRK1
NTRK3
NUCB2
OCLN
OGT
ONECUT1
OPA1
OPCML
OPTN
ORM1
OSM
OTOF
P2RX2
P2RX5
PAK6
PAK7
PAPPA
PARK2
PARK7
PARP1
PARVA
PAX2
PAX6
PBK
PCNA
PCSK1
PCSK2
PCSK9
PDGFRA
PDIA3
PDK1
PDK2
PDPK1
PEBP1
PECAM1
PF4
PFN2
PGR
PGRMC1
PHB
PHB2
PI3
PIAS1
PIK3R2
PKLR
PKM
PLAUR
PLEC
PLG
PLIN3
PLK1
PLK2
PLTP
PMVK
PNPLA6
POMC
POR
POU2AF1
POU2F1
PPARA
PPBP
PPIA
PPIB
PPID
PPP1R12A
PPP1R1B
PPP2CA
PPP3R1
PPT1
PRDX1
PRDX3

Antibody Gene Index (Continued)

PRDX4	RPS6KA5	SKAP1	SOCS3	TEC	TNXB	UPF3B
PRDX5	RRM2	SKP2	SOD1	TF	TOLLIP	VCAM1
PRDX6	RTN3	SLC10A1	SOD2	TFAM	TP53	VCAN
PRKAB2	RTN4	SLC12A1	SOD3	TFF1	TP53BP1	VCL
PRKCA	RUNX1	SLC12A2	SOX2	TFPI	TP63	VCP
PRKCE	RUNX3	SLC12A3	SP3	TFPI2	TP73	VDAC1
PRKCI	S100A6	SLC16A1	SP5	TFRC	TPM1	VDR
PRLR	S100A8	SLC16A3	SPARC	TGFBR1	TPP1	VEGFA
PRNP	S100A9	SLC16A4	SPARCL1	TGM2	TPSAB1	VIL1
PROC	SCG3	SLC1A3	SPHK1	TH	TRAF2	VIM
PROM1	SDC2	SLC1A4	SPI1	THBS2	TRAF4	VIP
Protein SSX2	SDC3	SLC22A2	SPN	TICAM1	TRAF6	VTN
PSEN2	SDC4	SLC22A6	SPP1	TIMP1	TRAM1	VWF
PTGS1	SDHA	SLC2A1	SPTLC1	TIMP4	TRAP1	WAS
PTGS2	SDHB	SLC2A2	SQSTM1	TJP1	TREM1	WDR83
PTH1R	SDHC	SLC2A4	SRC	TJP2	TRIB2	WISP1
PTN	SELE	SLC2A5	SRSF1	TJP3	TRIM28	WNK1
PTP4A2	SELENBP1	SLC2A8	SSR3	TLN2	TRIM33	WNT2B
PTPN11	SELL	SLC30A4	SST	TLR5	TRPC3	WNT5A
PTPN2	SELP	SLC4A1	SSTR1	TLR7	TRPC6	WNT7A
PTPRC	SERPINA1	SLC6A1	STAT1	TMEM173	TRPM2	XAF1
PTPRF	SERPINA3	SLC6A4	STAT3	TNC	TRPM4	XBP1
PXN	SERPINA4	SLC9A1	STAT5B	TNF	TRPM5	XCL1
RAB13	SERPINA6	SLC9A2	STAT6	TNFAIP1	TRPV1	XIAP
RAB5A	SERPINB2	SMAD1	STIM1	TNFAIP8L3	TRPV2	XPO1
RAB8A	SERPINB5	SMAD2	STK11	TNFRSF10B	TSC1	XRCC1
RAD51	SERPINH1	SMAD2/3	STMN1	TNFRSF11A	TSC2	XRCC3
RAG2	SF1	SMAD4	STRAP	TNFRSF1A	TSHR	XRCC4
RBBP4	SFN	SMAD5	STXBP1	TNFRSF1B	TTR	XRCC5
RBL1	SFRP4	SMAD7	SULT2A1	TNFRSF4	TXNRD2	XRCC6
RBP2	SFTPA1	SMARCB1	SULT2B1	TNFSF10	UBA3	YBX1
REL	SFTPA1/2	SMC3	SYNPO	TNFSF13B	UBB	YES1
RELA	SFTPD	SMN1/2	SYP	TNFSF8	UBD	YIPF3
RETN	SGK1	SMYD3	TAB2	TNN	UBE2Q2	ZAP70
RFC1	SHC1	SNAI2	TAP1	TNNC1	UCHL1	ZBTB7A
Rgs3	SHH	SNAP23	TBC1D4	TNNT1	UHRF1	ZP2
RNF2	SIRT2	SNAP25	TBK1	TNNT2	UHRF2	ZWINT
ROCK2	SIRT6	SNRPN	TCF7L1	TNNT3	ULK3	
ROR1	SIRT7	SOCS1	TCP1	TNR	UPF1	

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