Immunohistochemistry
Principles, uses and methods
**PRINCIPLES OF IMMUNOHISTOCHEMISTRY (IHC)**

Immunohistochemistry (IHC), or immunohistochemical staining, is a technique which employs antibodies to detect antigens in cells within a tissue section. This application is used to locate specific antigens in tissue sections with labeled antibodies based on antigen-antibody interactions. The immune reactive products can be visualized by a marker including fluorescent dyes, enzymes, radioactive elements or colloidal gold. The IHC principle has been known since the 1930s, but it was not until 1942 that the first IHC study was reported, where scientists used FITC-labeled antibodies to identify Pneumococcal antigens in infected murine tissue. Since then, advancements in protein conjugation, tissue fixation methods, detection labels and microscopy have allowed immunohistochemistry to become an essential tool in diagnostic and research laboratories.

**COMMON USES OF IHC**

Immunohistochemistry is commonly used by clinicians to detect and diagnose abnormal cells found in disease states such as cancer. Such biomarkers are specific to the disease state and are characteristic of particular events such as cell death, apoptosis or proliferation, which give rise to the abnormality.

Immunohistochemistry can also be used:

1. As a predictor for treatment, prognosis and outcome. For example Trastuzumab inhibits HER2 which is overexpressed in some but not all breast cancers. It is therefore not advisable to administer Trastuzumab to HER2 negative breast cancer patients. IHC can be used by clinicians to confirm overexpression of HER2 before administration of Trastuzumab.

2. During basic research, to evaluate the location and colocalization of proteins within a cell, for instance in the nucleus, cytoplasm or membrane.

*Figure 1. Immunohistochemistry image of a liver section stained for CD31*
FIXATION

The principle of IHC tissue fixation is to maintain tissue structure and retain antigenicity. Users must be sure to preserve the readable tissue architecture and cell morphology, otherwise the localization of immune reactive products cannot be recognized. However, if the tissue is over-fixed, the antigenicity will be diminished or even completely extinguished thus resulting in false negative staining.

There are a broad array of fixative methods commercially available. Researchers may choose appropriate fixatives based upon the tissue types and the requirement of individual experiments. However, we must iterate that there is no one universal fixative that is ideal for the staining for all antigens. In general, most antigens can be successfully identified in acetone-fixed tissue sections. Formalin-fixed tissue sections are also popularly used in IHC because formalin-fixed paraffin-embedded tissue can be stored for many years. It is very convenient to use archived paraffin-embedded tissue to carry out IHC. In recent years the use of antigen retrieval techniques has increased the use of formalin fixation and paraffin-embedding as a more commonly used fixation method.

ANTIGEN RETRIEVAL

After fixation, the epitopes may be cross-linked and covered making it difficult for antibody-binding. By pre-treatment with antigen retrieval reagents or procedures, investigators can re-open the cross-linked epitopes so that antibodies can easily bind to target antigens. Several approaches to antigen retrieval have been widely published, including heat and enzyme retrieval. Commercially available reagents are also available from multiple sources.

IHC METHODS

Blocking

Several endogenous substances may interfere with IHC results, such as endogenous peroxidase, fluorescence, antibody binding capability or biotin. Therefore, blocking the endogenous material prior to staining is crucial in IHC to avoid false positive staining.

Visualization of the Antibody-Antigen Complex

There are several potential options available to visualize the antibody-antigen complex in immunohistochemistry:

1. By directly conjugating an enzyme such as horseradish peroxidase (HRP) or alkaline phosphatase (AP), which can catalyze a color-producing reaction
2. By directly conjugating a fluorophore such as rhodamine or fluorescein
3. Indirectly through the use of a secondary antibody conjugated either to an enzyme or a fluorophore

ANTIBODIES

The specificity of an antibody refers to its ability to recognize a specific epitope in the presence of other epitopes. The use of an antibody with high specificity will result in less cross-reactivity. With respect to native protein antigens, the binding affinity of most antibodies is influenced by conformational determinants, and antibodies may not bind the same protein in a denatured state. This characteristic is particularly true of mAbs, which target a single epitope. Conformation may be altered by any number of factors, including association with other proteins, post-translational modification, temperature, pH, salt concentration, and fixation. The impact of conformational change is of less concern when using pAbs. pAbs recognize multiple epitopes, some of which are likely to be linear, and conformational changes may not influence all epitopes to the same degree.
The measure of the binding strength of an antibody for a monovalent epitope is referred to as affinity. Precise affinities can be ascertained for mAbs because of their homogeneous nature, however affinity can only be estimated with pAbs because they are composed of numerous antibodies of varying affinities. Antibodies with high affinity bind larger amounts of antigen with a greater stability in a shorter time than those with low affinity and are preferable for immunohistochemical techniques. The use of pAbs can result in nonspecific background staining, however affinity purification using the desired antigen immobilized on a solid support can be used to minimize or eliminate the problem.

Monoclonal antibodies are specific for a single antigenic epitope and are thus considered to be more specific to the target antigen than polyclonal antibodies. Monoclonal antibodies generally have a higher affinity than polyclonal antibodies and thus are usually a better choice for IHC techniques. Polyclonal antibodies are a heterogeneous mix of antibodies that recognize several antigenic epitopes and are less likely to be affected by conformational changes, however they usually demonstrate a lower affinity thus are less preferable for use in IHC.

INDIRECT VS. DIRECT DETECTION METHODS

The label in an immunoassay provides either ‘direct’ or ‘indirect’ detection of the antigen. With indirect detection, the label is covalently attached to a secondary antibody, which is allowed to bind to the primary antibody in the immunoassay. Alternatively, using direct detection, the label is attached via a covalent bond to the primary antibody.

In the case of indirect detection, the assay comprises two distinct parts. First, the unlabeled antibody is incubated with the tissue section. If the antigen is present, the primary antibody will bind to it. Excess unbound primary antibody is then washed away and a labeled secondary reagent is added. After a period of incubation, excess secondary reagent is washed away and the amount of label associated with the primary antibody (i.e. indirectly via the secondary reagent) is quantified. The label usually results in the production of a colored substance or an increase in the amount of light emitted at a certain wavelength, if the antigen is present. In the absence of antigen there is no binding of the primary antibody and no binding of the secondary reagent, and thus no signal.

With direct detection, the prior covalent attachment of the label to the primary antibody means that only a single incubation step with the antigen is required and only a single round of wash steps, as opposed to two rounds of incubation and wash steps with indirect detection. The assay simplification that is afforded by direct detection tends to decrease assay variability and improve data quality.

Some of the pros and cons of direct/indirect detection methods are given in table 1.

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<tr>
<th>METHOD</th>
<th>PROS</th>
<th>CONS</th>
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<tbody>
<tr>
<td>Direct</td>
<td>• Quick methodology since only one antibody is used</td>
<td>• Immunoreactivity of the primary antibody may be reduced as a result of labeling</td>
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<tr>
<td></td>
<td>• Non-specific binding of secondary antibody is eliminated</td>
<td>• Little signal amplification</td>
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<tr>
<td>Indirect</td>
<td>• Sensitivity is increased because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody, allowing for signal amplification</td>
<td>• Non-specific binding may occur with the secondary antibody</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Extra incubation and wash steps are required in the procedure</td>
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Table 1. Advantages & disadvantages of direct vs. indirect staining.

Despite the potential advantages of direct detection, many immunoassays today still employ the principle of indirect detection. Undoubtedly the main reason for this is that direct labeling of primary antibodies is relatively complicated and indeed, historically, antibody labeling has been carried out only by those with specialist knowledge of chemical-modification techniques.
DIRECT DETECTION DATA

Lightning-Link® antibody labeling kits can be used to directly conjugate antibodies for IHC to HRP or other labels. The IHC data below shows that it is possible to obtain high quality staining whilst avoiding the use of secondary antibodies, therefore saving significantly in time and reagent costs.

![Unconjugated L26](image1)

![HRP conjugated L26](image2)

**Figure 2.** Anti-human CD20 (clone L26) was conjugated to HRP using Lightning-Link® kits, and used in IHC in comparison to a traditional indirect technique.

Figure 2 shows a comparison of the anti-CD20 mAb L26 staining of paraffin-embedded bone marrow, with Dako Envision as the detection system, and the same antibody directly labeled with HRP using Lightning-Link®. The use of the directly labeled antibody at different concentrations, and with different HRP labeling ratios, also shows clear staining of the B lymphocytes, without background staining.

In figure 3, mouse anti-human CD20 was conjugated to HRP using Lightning Link® kits, and rat anti-human CD3 was conjugated to Alkaline Phosphatase using Lightning Link® kits. Both antibodies were then used simultaneously to stain formalin fixed paraffin embedded sections of human lymph node. CD20 positive cells were visualized with DAB and are stained brown, and CD3 positive cells were visualized with Fast Red and are stained red.

![Dual staining of CD3 and CD20](image3)

**Figure 3.** Dual staining of CD3 and CD20 with directly conjugated antibodies using Lightning-Link® kits.

Direct labeling of primary antibodies with Lightning-Link® kits enables very simple dual color staining in IHC, which is otherwise a highly complex technique.
THE COMPLEXITY OF IMMUNOHISTOCHEMISTRY - REAGENT SOURCING AND AVAILABILITY

The scientist has a vast array of products and suppliers to choose from when selecting immunohistochemistry reagents as there are a large number of antibody manufacturers. However, it is an expensive task for the commercial market to provide all antibodies pre-labeled. Despite this, scientists still need access to such conjugates to advance their scientific research and so it is necessary for the end user to label commercially sourced antibodies in a cost effective manner whilst simultaneously overcoming the difficulties of conventional labeling approaches such as antibody loss.

Lightning-Link® - the world’s easiest antibody labeling technology

Lightning-Link® antibody labeling kits enable the direct labeling of antibodies and proteins with only 30 seconds hands-on time.

Furthermore there are no column separation steps required, so there is no loss of material.

The Lightning-Link® labeling process is outlined in figure 4.

Figure 4. Schematic representation of the Lightning-Link® labeling process.

During the Lightning-Link® conjugation reaction the purified antibody is transferred to a vial containing the label and the linking chemistry, which becomes activated upon dissolution. As there are no purification or separation steps (by products of the reaction are completely benign), antibody recovery is close to 100%. The simplicity of the approach means that the labeling procedure can be completed in less than 30 seconds. A timed demonstration of the antibody labeling process can be seen in our video. The labeling chemistry targets primary amine groups found on lysine amino acids, and so any antibody or protein, irrespective of isotype or species, can be labeled.

More benefits include:

- Save time - 30 seconds hands-on time! No tiresome extra wash and incubation steps
- 100% recovery - no antibody/protein loss
- Covalent bond- highly stable conjugates
- Fully scalable (10ug to 1g or more)
- Consistent high quality, excellent batch-to-batch reproducibility
- Large number of labels available
- Long shelf life
- Reliable: over 300 references

There are over 40 available labels in our range of Lightning-Link® kits. This unique technology allows the end user to develop a panel of labeled antibodies using commercially sourced, or user-generated, unlabeled antibodies.
Furthermore, there are many benefits associated with direct labeling of a primary antibody:

1. Eliminates the need for the use of secondary antibodies, thus reducing the number of incubation and wash steps - saves both time and money.

2. With indirect detection, cross-species reactivity of secondary antibodies is often a problem. In experiments which use several antibodies simultaneously, cross-species reactivity is not an issue as the primary antibodies can be directly labeled with different dyes.

3. Commercial sources often do not have the correct antibody conjugated to the required label. By direct labeling of the antibody of interest yourself, this hurdle is easily overcome.

Additional information can be found in the ‘Guide to Labeling Your Primary Antibody’.

The antibody labeling kits come in two formats - Lightning-Link® and Lightning-Link® Rapid, with incubation times of 3 hours and 15 minutes respectively.

IHC REPORTERS

In order for the antigen–antibody immunoreaction to be seen under the microscope, the antibody must be labeled with an enzyme, fluorophore, colloidal gold or biotin. An enzyme label can be visualized using the light microscope by means of enzyme histochemical methods via chromogenic reactions. A fluorophore label can be directly visualized using a fluorescent microscope. Electron-dense labels such as colloidal gold are visible in the electron microscope without further treatment.

ENZYMES VS FLUOROCHROMES

In enzyme-histochemical chromogenic reactions, a soluble colorless substrate is converted into a water-insoluble colored compound, either directly or in a coupled reaction. Histochemical detection of peroxidase is based on the conversion of aromatic phenols or amines, such as diaminobenzidine (DAB), into water-insoluble pigments in the presence of hydrogen peroxide (H2O2). Alkaline phosphatase catalyzes the hydrolysis of a variety of phosphate-containing substances in the alkaline pH range. The enzymatic activity of alkaline phosphatase can be localized by coupling a soluble product generated during the hydrolytic reaction with a “capture reagent,” producing a colored insoluble precipitate.

Fluorescence microscopy is based on the phenomenon by which absorption of light by fluorescent molecules called fluorescent dyes or fluorophores (known also as fluorochromes) is followed by the emission of light at longer wavelengths, usually in the visible region of the spectrum. Fluorochromes can be visualized by fluorescence microscopy using special filter sets.

Colloidal gold is a suspension (or colloid) of sub-micrometer-sized gold particles in a fluid, usually water. A colloidal gold conjugate consists of gold particles coated with a selected protein or macromolecule, such as an antibody, protein A or protein G. Because of their high electron density, the gold particles are visible using the electron microscope without further treatment. For electron microscopical immunolabeling, the gold particles are manufactured to any chosen size from 6 to 25 nm. The size of the gold particles may be enlarged with subsequent use of a silver enhancement technique. Silver enhancement occurs during the reduction of silver from one solution (the Enhancer) by another (the Initiator) in the presence of gold particles. The reduction reaction causes silver to build up preferentially on the surface of the gold particles. Enlarged in this way, the gold particles can be seen at much lower magnifications, and even using the light microscope. In the light microscope, the intense brown/black stain produced by silver enhancement of the gold signal gives a greater overall sensitivity and far greater resolution than other immunocytochemical methods, whereby tissues can be counterstained with all the usual staining procedures.

Expedeon also offers gold nanoparticle labeling kits for your convenience.
COUNTERSTAINS

After immunohistochemical staining of the target antigen, a second stain is often applied to provide contrast that helps the primary stain stand out. Many of these stains show specificity for discrete cellular compartments or antigens, while others will stain the whole cell. Both chromogenic and fluorescent dyes are commercially available for IHC to provide a plethora of reagents to fit every experimental design.