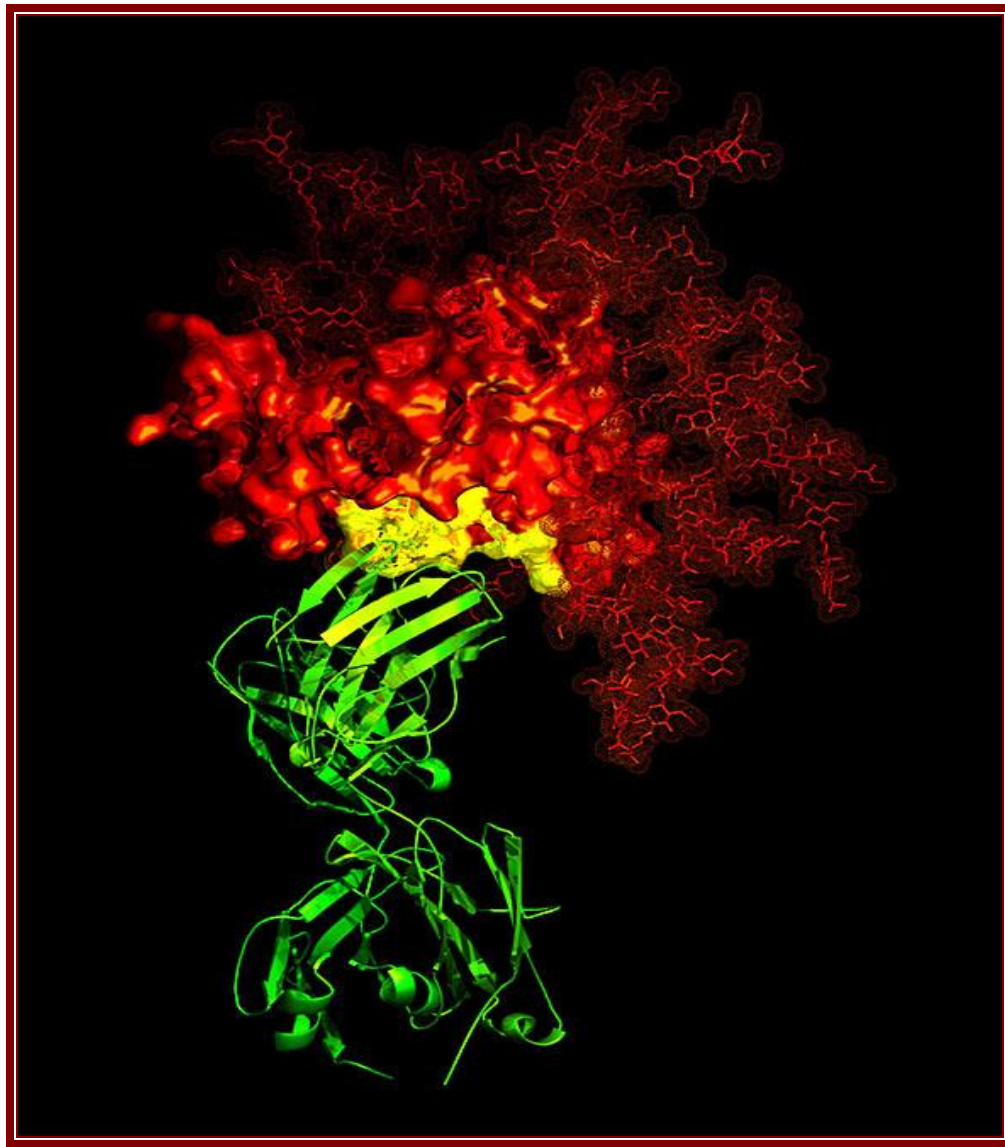
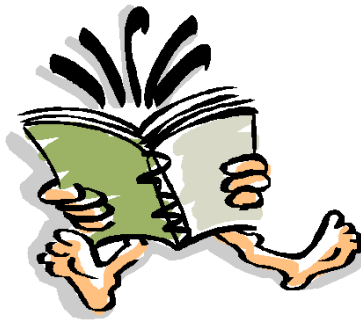


Principles of Immunohistochemistry  
Queen's Laboratory For Molecular Pathology





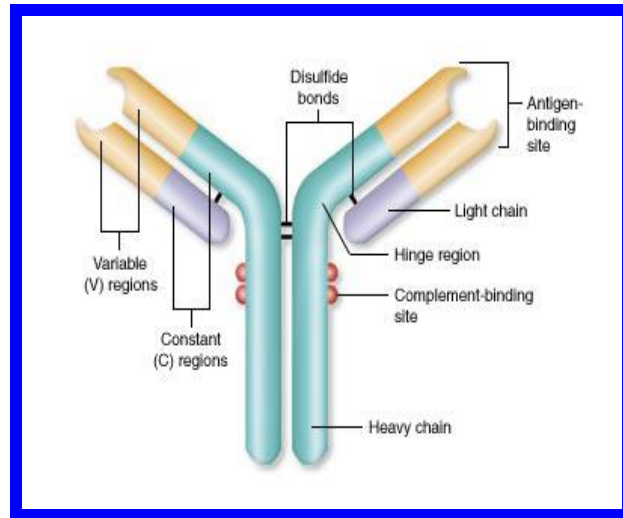
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## Principles of Immunohistochemistry

This pamphlet provides a general overview of the basics of IHC, giving some insights into the operating principles of antigen-antibody interactions and IHC protocols that take advantage of these interactions.

Immune cells, or B cells, produce antibodies against targeted proteins. These antibodies then can recognize, and bind to, those proteins, called antigens. More specifically, they have an affinity for certain sites on those antigens, called epitopes.



Antibodies are also called immunoglobulins, abbreviated Ig. There are different classes, or isotypes, of Ig molecules, designated with a third letter, such as IgG, or IgA. Different isotypes of immunoglobulins perform different functions, in different places in the body. IgG provides the majority of antibody based immunity against invading pathogens.

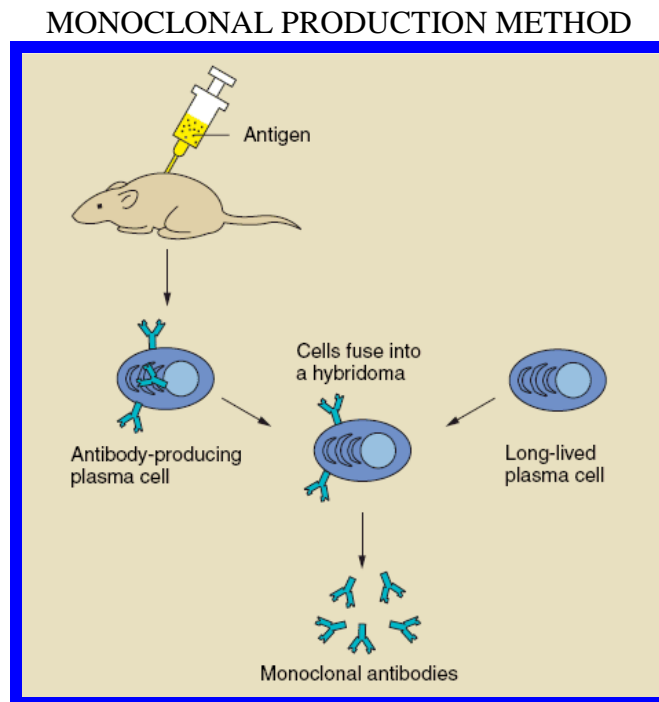
### Polyclonal and Monoclonal antibodies

When the immune system detects the presence of a foreign body, such as a virus, many B cells will produce antibodies against it. The different B cells will target different sites on the antigen, producing a mix of antibodies, all against that virus, but specifically against many different epitopes on that antigen. This produces a polyclonal antiserum where “polyclonal” denotes the mixed population of Ig molecules.

In contrast, a monoclonal antibody preparation contains a single antibody with specificity to one epitope on the antigen molecule. This is achieved by fusing B cells from the spleen of an immunized animal with immortal myeloma cells, and growing clones from the single parent cells on microtitre wells. The most productive clone, or hybridoma will be selected for production and purification of a single epitope antibody.

Typically monoclonal antibodies have been mouse antibodies, however advances have seen the production of many rabbit monoclonals lately.

Monoclonals and polyclonals have their advantages and disadvantages. Polyclonals are cheaper, because they are cheaper to produce. They are typically more sensitive as they are directed against many sites on the antigen. This will increase the chance that some of the antibodies will survive adverse conditions and complete binding to the antigen. Because of this robust nature of the interaction, polyclonals in general can be used at higher dilutions, as detection of the epitope in sensitive assays is more assured. Monoclonals will be more expensive, and may be more vulnerable to adverse conditions, due to their narrow specificity. But this specificity is very desirable for highly focused, low background immunostaining.

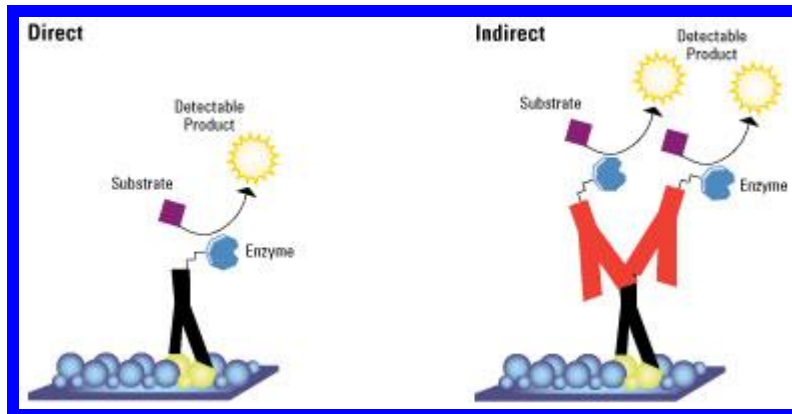


### Direct and Indirect IHC

Immunohistochemistry takes advantage of antigen-antibody affinity through its ability to identify and localize proteins of interest via detection with labelled conjugates. In the simplest example, a primary antibody (first in order of use, directed to the antigen epitope) which has had a label attached, is allowed to bind. The antibody, and thus the antigen on the tissue surface, is then detected through a series of treatments, which will visualize the label for analysis and quantification. This is called direct IHC.

One other important characterization of an antibody that we must consider is the animal that the antibody was developed in. If an antibody against, say, vimentin has been produced in mouse, this is termed “mouse anti vimentin”. This distinction is important when considering another binding assay, called indirect IHC. Indirect IHC involves using an intermediary between the primary antibody and the detection system.

In this method, the primary antibody, without label, is bound to the antigen as in the direct method. But then a second antibody is applied. This antibody will have been developed against mouse antibodies in general, and so is termed “anti-mouse”. Because this second antibody (surprisingly called the secondary) is anti-mouse, it will bind to any mouse derived primary antibody. This secondary antibody will be labelled, which then can be detected as before. Because the primary antibody is being indirectly detected through the use of an intermediate Ig molecule, this is termed the indirect method.



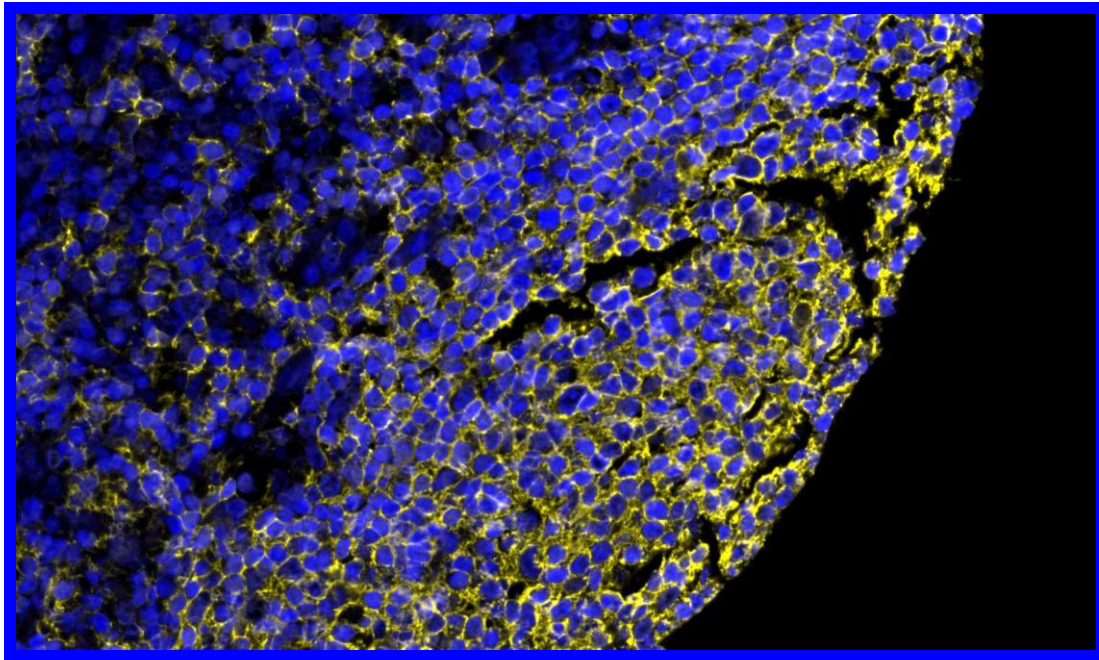
The direct method of IHC is simple and efficient, but the indirect holds some major advantages. With the direct method, you need specific labelled primary antibodies for every protein you want to investigate. But if you are using the indirect method, one labelled anti-mouse secondary will work with all mouse derived primaries. One anti-rabbit secondary will work with all rabbit derived primaries, and so on, thus reducing the number of labelled Ig molecules needed down to one for each type of antibody. A further advantage lies in the fact that each primary antibody will usually bind at least 2 labelled secondaries, thus doubling (at least) the signal of the assay. Generally the indirect method is seen as about 10 times more sensitive than the direct.

### Labels

A variety of enzymatic and fluorescence type labels may be used. For our purposes here I will cover a few of the more commonly used ones. Enzymes such as horseradish peroxidase use chromogens that produce a precipitate visible with a normal light microscope. These are termed “brightfield” images. They are stable, permanent, and allow counterstaining so that the surrounding tissue can be viewed to give context to the antigenic site.

Fluorescent labels produce a bright colour at a specific wavelength, allowing the antigen-antibody complex to be viewed in “darkfield”, on a fluorescence microscope. In a blacked-out field, only the label or labels used show up.

This can result in dramatic images of specific aspects of tissue morphology, including pronounced emphasis on antigen site to site comparisons. For example, below is an image in darkfield using 2 fluorescent labels. One label has targeted cellular membranes, the other being a nuclear stain. In combination, the two produce yellow-green rings around blue nuclei. In this simple example, it may be that the protein being expressed in the membrane, and visualized with the yellow-green fluor, is not expressed in all cells. If this were the case, we could compare numbers of cells (blue nuclear stain) with and without membrane staining (yellow-green), giving us data on percent of cells expressing.

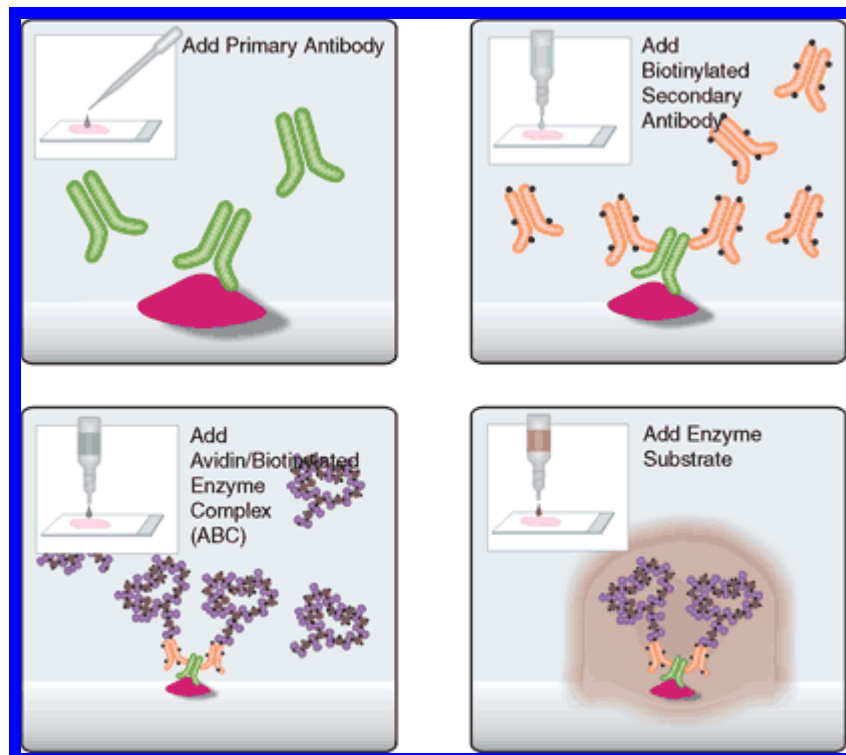


Brightfield images are permanent, and allow counterstaining, but for quantification, they provide an image which digitally is mostly positive or negative. It is difficult to measure a range of signal strength as the chromogen tends to be opaque. There also are indications that during the reaction the chromogen precipitate can diffuse away from the epitope site, but for most purposes this does not pose a significant problem. Fluorescent labels are not permanent. They are subject to fade, and burn-out on the scope. A fluorescent stain should be digitized as soon as possible to permit long-term storage and potential analysis of the optimal image data. But to their advantage, they can be measured for their intensity, giving a more accurate and gradable range of signal strength.

## Detection

Earlier I summarized detection as a series of treatments. I can expand on these now. The first label I mentioned was an enzymatic label using horseradish peroxidase (HRP). We've seen how the first steps in this process involve getting the labelled secondary conjugated to the primary antibody. The purpose for the next series of steps is to put a chromogen in place that will show up in brightfield. One of the most common methods is to use the HRP enzyme (label) to catalyze a DAB substrate, creating a reaction product precipitate at the site which is visible by microscopy. The signal of this interaction can be augmented by inserting steps in the chain which take advantage of the strong biotin-avidin affinity, or the biotin-streptavidin affinity. DAKO's LSAB kit utilizes streptavidin in this fashion. In this procedure, the secondary antibody is not labelled with HRP, but with biotin. The secondary is now a biotinylated antibody. This biotinylated molecule binds to the primary, and then is bound to several HRP labelled streptavidin molecules. This technique places many more HRP molecules into the conjugate, increasing the sensitivity of the assay many fold. Once the HRP molecules are bound, they can catalyze the DAB substrate-chromogen into a visible precipitate.

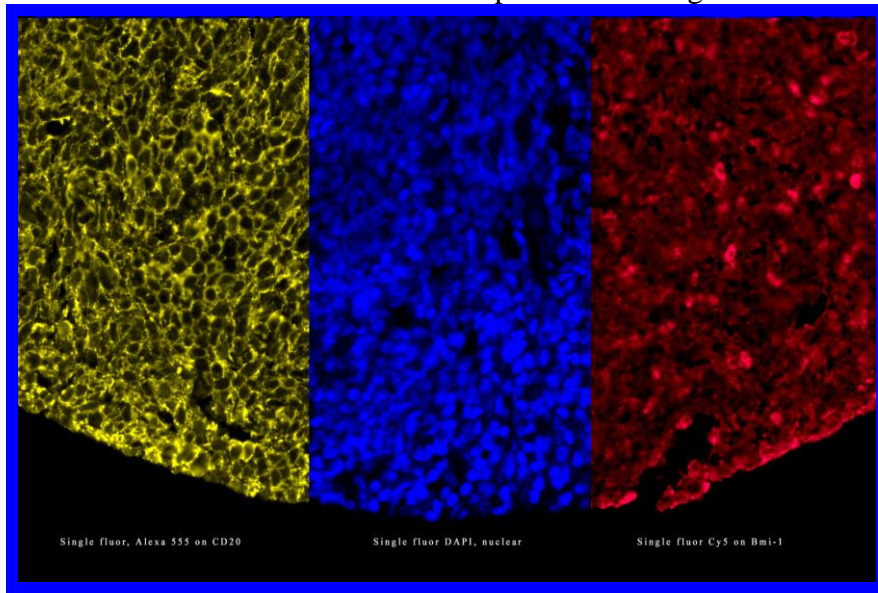
### AVIDIN-BIOTIN ASSAY



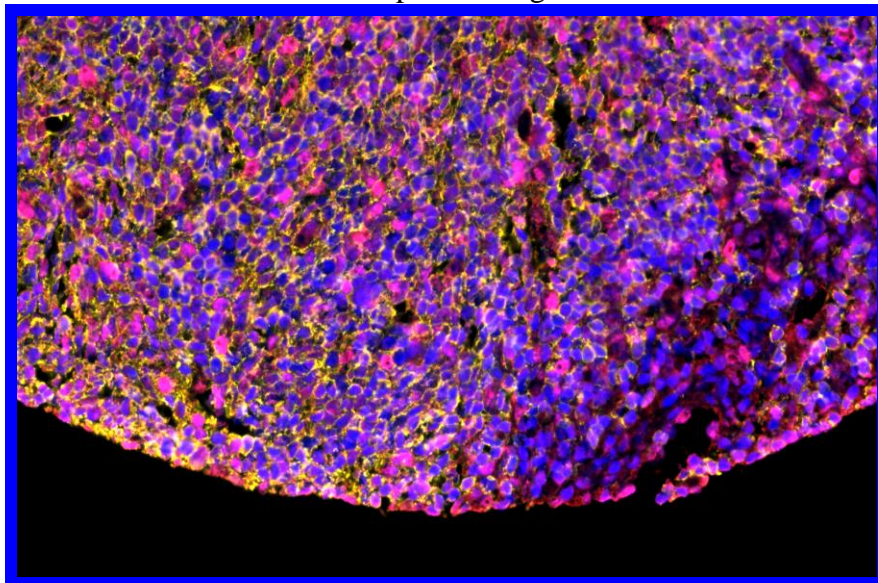
HRP is the most widely used enzyme label, but others, such as alkaline phosphatase, or glucose oxidase, can be used. This provides a wider range of conditions available to choose from, and also allows the possibility of dual staining on one tissue section.

Fluorescent labels have the advantage of being easier and quicker to use, as they require fewer steps. Typical fluorochromes are Texas red, FITC, DAPI, and a variety of Alexa fluorochromes, such as Alexa 488, which, for example, fluoresces green with a maximum emission at an excitation wavelength of 488. Once bound, detection simply involves observing the section on a fluorescent microscope at the correct wavelength. Dual and triple stain images can be produced by scanning the section at each required wavelength, producing multiple images that can be superimposed one on another, resulting in a multiple stain image. Below are 4 images of the same section. This section has been double labelled with Cy5 on mouse Bmi-1 (red), and Alexa 555 on rabbit CD20 (yellow-green). A 3<sup>rd</sup> fluorochrome, DAPI, (blue) has been added in the cover slip medium. The panel of 3 images shows the individual fluorochromes, the fourth image shows all the colours together.

One section viewed at 3 separate wavelengths



Composite Image





In cases where the primary antibody signal is weak, amplification can be achieved with a DAKO Envision system. This kit from DAKO utilizes a long chain polymer conjugated with an Ig fragment that will be anti mouse, or anti rabbit, allowing for binding to the primary. The polymer is HRP labelled, which then allows for the usual DAB detection. The advantage here is that the polymer will have about 14 HRP molecules on each chain, providing a substantial amplification of signal. It is important to note that a weak signal may not be due to a poorly performing primary antibody. Weak signal may result from low quantities of antigen on the tissue, or non-optimal assay conditions. The first response to boosting a weak signal may not necessarily be amplification.

### Tissue preparation

The binding of the primary antibody to the antigen epitope requires the epitope to be in a condition that maximizes availability for reaction. Reduced accessibility of the primary antibody to the binding site will weaken or eliminate signal. Preparing the tissue to receive the primary antibody is an integral part of immunostaining, and is referred to as antigen retrieval, or A/R. Most tissue used in immunostaining has been formalin fixed and paraffin embedded. This treatment degrades the antigen to the point where, for many antibodies, the tissue requires one of several treatments. Heat induced epitope retrieval (HIER), is most commonly used. This involves an incubation period at or around 95° C. This can be achieved with steam bath (rice cooker), or microwave treatment, or pressure cooking. Buffers used here will be pH specific, as some antibodies require low pH (citrate, ~6) antigen treatment, and some require high pH (EDTA, ~8). Certain companies, such as DAKO, provide these buffers. They are quite expensive, but can be used many times. Other antibodies perform optimally with protease treatment, such as Proteinase K. Frozen sections do not require such drastic A/R protocols, as the antigens are still viable.

Section prep can be responsible for signal loss as well. High temp drying onto the glass slide must be monitored closely. 58° C to 60° C should be the operating range, for an incubation period of 2 hours. Sections should be as fresh as possible. Whenever possible the section should be stained within one or two days of mounting and drying. Some antibodies show reduced signal on sections that are only 3 or 4 days old. I should note here that some antibodies are more robust, and may provide a strong response on sections that are months or even years old. It is good policy to simply keep your sections as fresh as you possibly can, whichever antibody you are using.

During the IHC assay, the tissue section goes through multiple buffer exchanges and rinses. It is important that these exchanges be performed in an orderly and timely fashion. If the section dries out for just a moment, the assay is ruined.

Poor formalin fixation will also impact quality of signal. Most labs doing IHC will not have direct control over the fixation of tissues, but you should be aware that this can impact signal strength. An example of a poorly fixed piece of tissue would be where the formalin has not penetrated completely, producing a “halo” effect. This will result in strong signal around the edges of the section, with a weaker, faded response towards the interior.

## Blocking

IHC assay conditions are designed to optimize binding of the various specific components of the conjugates we are trying to assemble. Any other unintended binding is termed non-specific, and will obscure true binding, and suggest false positives. In order to install the correct blocking measures in your assay, you must be aware of the properties and actions of each component.

When applying primary and secondary antibodies to the surface of the tissue, the antibodies will bind to non-specific sites by hydrophobic and electrostatic forces, generally with low affinity. This binding can be blocked in a variety of ways. A commonly used method is to wash the tissue, prior to application of the antibodies, with normal serum from the species of the secondary antibody.

This will mask non-specific reactive sites while allowing specific binding to take place. Use of normal sera from the species the primary antibody was generated must not be used, as the secondary will bind all across the tissue in this case.

Another approach, one used here in the QLMP lab, is to use 1% BSA (Fraction V) in solution. This is a universal block, binding to many sites on the tissue. When the primary antibody is applied, it has a stronger affinity for its target epitope, and so outcompetes the buffer block for the site. As the primary has no greater affinity than the buffer block for the non-specific sites, it does not displace them, reducing non-specific staining. For optimal conditions the dilution buffer used for the antibodies should contain the same type of blocking buffer used in the assay. This acts as a stabilizer.

The most common enzyme used in IHC assays is HRP. This peroxidase catalyzes the DAB substrate-chromogen to produce a visible precipitate. We want this to happen only on our targeted conjugate. However, tissues contain endogenous (naturally occurring) peroxidases that will also drive the reaction. This must be blocked. Typically a 3% H<sub>2</sub>O<sub>2</sub> solution is applied after A/R, and prior to incubation with the primary. Alternate enzymes will require different blockers. For example, if using alkaline phosphatase, endogenous activity should be blocked with levamisole. H<sub>2</sub>O<sub>2</sub> degrades to water over time, so be sure your solutions are fresh. This applies to your BSA blocks as well.

As already outlined, many IHC assays utilize the affinity between biotin and avidin, or streptavidin. The biotin label on the secondary presents a binding site to a HRP labelled streptavidin molecule. The problem here is that some types of tissue, such as liver and kidney, have high levels of endogenous biotin. This endogenous biotin can be blocked with avidin/biotin washes, before the introduction of the conjugated streptavidin.

As a footnote to blocking issues, there is debate today as to whether or not blocking against non-specific binding is even necessary anymore. With increased specificity of today's antibodies, and higher quality reagents overall, some researchers feel background noise in most typical assays is low enough to justify excluding the use of a blocking step. This is a decision probably best left up to the individual researcher, as every antibody comes with its own set of requirements and characteristics needed for optimal performance. Certainly inclusion of appropriate blocking steps will do no harm.

## Rinsing

You might think variations in procedure here would be of little consequence, however there are some principles that should be observed. It is important to apply sufficient rinsing between each step, as excess remnants of the last incubation will interfere with the next component. As important as sufficient rinsing is, it is also important to be careful not to rinse too vigorously. It is possible to strip off previously applied components from the tissue, leaving the signal to appear blotchy or absent in random areas on the tissue. Follow a formal routine for rinsing, such as using a series of dips in rinse buffer combined with gentle washes from a transfer pipet. Avoid spraying rinse buffer directly at the tissue section. Flood the slide so the buffer washes over the section.

## Controls

When assessing the outcome of your immunostain assay, experimental results are meaningless unless they are judged in the context of appropriate controls. When applied and interpreted correctly these controls assure us that reagents and protocols were correctly applied, and performed as expected.

Many antibody producing companies will tell you which tissues act as positive controls for your antibody. Positive controls are tissues you run with the experimentals that have been proven to produce a signal if all is correct with the assay. If you run one experimental slide, and it is negative, it may be because the results are a true negative. But it may also be that the assay did not work properly, resulting in a failed stain. By including a positive control, you will know if your assay performed as expected. The internet site “Human Protein Atlas” is an excellent site for finding positive control tissue suggestions for many antibodies, and also for providing actual images showing how your antibody staining should look.

A positive control should be included in every assay, as errors and omissions easily slip in to these many-step protocols. We are all human. Positive control sections should be comparable to the experimentals in every way that you have control over. Age of section, drying protocols, fixation, etc. In general, normal tissue should be used instead of cancer samples. As a rule of thumb, the positive control should operate in a range that produces a medium to weak signal on the tissue. In this way, if the assay starts to produce variable results (due to fading antibody viability, old buffers, etc) you will see it in this kind of signal. If you are using a positive control that produces a heavy, dark stain, and the signal fades by, say, 20%, you probably would not notice it.

The next control you should include in your assay is the negative control. For this control, set aside one duplicate experimental slide. This slide will receive all reagents the other slides get, and go through exactly the same processes in all ways save one. Do not apply the primary antibody. Apply the buffer the antibody is usually applied in, just omit the primary. In this way the secondary has no target binding site, and this should result in no label on the slide, and thus no signal. If you still get signal, this tells you the secondary is finding non-specific binding sites, and some or all of your signal is false. Re-assessing your blocking strategies may be necessary.

If you cannot reduce this background noise to zero, you can at least compare the control with your experimental slides, to help identify true signal from false.

Another negative control involves omitting all antibodies, along with the enzyme label. If signal results, something in the tissue is catalyzing the DAB (such as endogenous peroxidases), and needs to be blocked. This control is not often performed.

One of the best controls will tell you if your primary antibody is truly specific for the antigen you are assaying. This involves “absorbing” the antibody, prior to application, with a purified solution of the reactive antigen, or peptide. In theory you will be applying an antibody that no longer has the ability to bind to the antigen epitope. Signal should be completely knocked out. When absorbing the primary, it may be possible that the peptide does not bind specifically, but still attaches in some way to interfere with binding when the antibody is applied. In this case you would get a false negative result. To control against this, you can absorb the antibody with a non-reactive peptide. You know this peptide will not be specific, so if staining is reduced or eliminated you are getting non-specific interference from the peptides.

The ideal results will be demonstrated by 3 outcomes: staining on the experimental, similar staining using the antibody absorbed with the non-reactive peptide, and no staining with the antibody absorbed with the reactive peptide. When you obtain these results you are assured your primary antibody is specific against the target antigen.

When preparing to absorb the primary antibody with the peptide, the 2 should be mixed in a ratio that has the peptide at a strength many times higher than the antibody. The usual suggestion is to use the peptide at a concentration of at least 10 times that of the antibody, on a molar basis.