Volume 57(1): 1–5, 2009
Journal of Histochemistry & Cytochemistry
http://www.jhc.org

PERSPECTIVE

A Guide to the Perplexed on the Specificity of Antibodies

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SUMMARY Many investigators are unaware of the potential problems with specificity of antibodies and the need to document antibody characterization meticulously for each antibody that is used. In this review, I consider the principles of antibody action and how they define a set of rules for what information should be obtained by the investigator before using an antibody in a serious scientific investigation. **(J Histochem Cytochem 57:1–5, 2009)**

KEY WORDS
immunohistochemistry
immunocytochemistry
controls

SINCE THE DESCRIPTION of indirect immunohistochemical (IHC) staining by Coons (1958), IHC staining has become a standard method used in most laboratories doing cellular or systems level localization of proteins and other cellular constituents. In fact, the methods have become so mundane that many current practitioners take for granted that an antibody that is sold to localize a particular molecular target will be both sensitive and specific. In the current era of very accurate DNA analyses by in situ hybridization, DNA chip analyses, and deep sequencing, it is often assumed that IHC has an analogous ability to identify molecular targets accurately.

Nothing could be further from the truth.

In fact, IHC methods remain as primitive, in terms of both sensitivity and specificity, as they were in the days when DNA sequencing was done by hand using sequencing gels. The fundamental principles on which antibody localization is based have not improved at all in the last two decades, and if anything, the slope occupied by IHC has become more slippery than ever.

In this review, I will first consider the basic physical chemical properties of antibodies that are responsible for whatever specificity and sensitivity they possess. I will then examine how modern advances have altered these fundamental principles. Finally, I will attempt to come up with a set of rules for establishing, as far as can be done, the specificity of an antibody preparation.

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Received for publication September 16, 2008; accepted September 16, 2008 [DOI: 10.1369/jhc.2008.952770].

Principles of Antibody Action

Antibodies are proteins in the immune globulin family that are produced by B-cell lymphocytes as part of the adaptive immune response. Immune globulins are coded by unusual genes, which contain a variable region that varies between B cells but remains the same for the entire life of the individual B cell (Neuberger 2008). The variable region of an immune globulin bestows on it the ability to bind to a specific molecular target, which fits the structure of the binding site. The specificity of immune globulin binding sites can be exquisite, recognizing an R vs S enantiomer of the same molecule or identifying proteins only when they are phosphorylated. On the other hand, a variable region that binds a common molecular motif may bind to many targets. In fact, the molecular motif that is recognized may be a function of tertiary folding, so that it need not even be a series of consecutive amino acids in a protein.

The binding affinity of an immune globulin for its target is also variable, depending on how well the target fits the variable region. Binding affinities in the range of 10^{-10}–10^{-11} M are common compared with the binding affinities of enzymatic sites, which are often in the range of 10^{-6}–10^{-8} M. This tight binding permits IHC staining, because the antibody can bind to its target so tightly that it can survive persistent washes, thus allowing the non-bound (or loosely bound) antibodies to be washed away.

When a molecule is present in a vertebrate animal that is not part of the "self," it is processed by antigen-presenting T cells. These present the antigen to B cells, which bind it, and the B cells are activated to produce and secrete their own immune globulins. Immune globulins can be in several classes (IgG, IgM,

 0022-1554/08/$3.30

IgA, IgE), which are produced at specific times and locations. However, each B cell can secrete only a single type of immune globulin, with only a single sequence at its variable site (Neuberger 2008). The antibody molecules produced by a single B cell are therefore identical. When a B cell is activated, it begins dividing, and all of the daughter cells in that clone also produce the same antibody. By fusing individual antibody-producing cells with antibody-producing myeloma cells, individual cells can be immortalized, so that they divide into colonies of "hybridoma" cells, all of which produce the same, identical immune globulin, with the same variable region. These monoclonal antibodies have the property that they will only bind to molecules that bind that single variable site. Although this relationship imparts specificity to the interaction, it is possible that the variable site may bind to a variety of different targets, particularly when tested in different tissues, and that these may be quite different from the molecule against which the antibody was raised.

A natural immunogen usually contains several molecular components that excite antibody responses. The resulting antibodies will each be caused by a clonal expansion of one or more B cells. Thus, naturally occurring antibody responses are termed "polyclonal" because there are usually many clones that will bind the target. Each clone (a single variable region immune globulin) will bind to a single molecular target on the molecule, called an epitope, but the epitopes recognized by different antibody clones within a polyclonal antiserum may overlap.

Although, in principle, an antibody can be made against any molecule, which need not even be organic, in general, most antibodies used in biological experimentation are made against a globular, aqueous phase protein or peptide. Although the conformation of the protein may not be identical with the native protein (which may be further modified in various ways), the antibody clones that are derived will recognize epitopes that are exposed on the surface of that protein in the aqueous state.

However, for IHC, most tissue are fixed, usually by aldehydes. Aldehyde fixation is based on a chemical reaction in which a carbon that is double bonded to an oxygen undergoes a reversible reaction with a molecule of water in which it instead becomes single bonded to two reactive oxygen atoms, each of which is bonded to a hydrogen (Fox et al. 1985). In basic environments, the hydrogens are stripped off, and the oxygen molecules may react with sites on different amino acids, thus cross-linking the amino acids in the peptide chain and changing the conformation of the molecule. For this reason, fixation may be improved by perfusing initially with a low pH solution of formaldehyde, to allow better tissue penetration, and then shifting to a high pH solution that makes the formaldehyde that has entered the tissue more reactive. Formaldehyde has a single aldehyde group and cross-links two closely spaced sites. Glutaraldehyde has two aldehyde groups, and each binds two closely spaced sites, but the two ends of the molecule bind at different sites in the protein, because they are separated by three additional carbon atoms. For this reason, glutaraldehyde usually causes greater molecular deformation, and although fixation and preservation of structure is better (e.g., for electron microscopy), antibodies that recognize a protein in globular form (a Western blot) or in formaldehyde-fixed material may not do so in glutaraldehyde-fixed tissue.

Modern Improvements in Antibody Production and IHC

Monoclonal Antibodies vs Polyclonal Antisera

As indicated above, access to monoclonal antibodies has provided us with antibodies that are pure reagents. The monoclonal antibodies are derived from hybridoma cells, which are grown either in culture or by injecting them intraperitoneally in a host animal. When the hybridoma cells are grown intraperitoneally, the host animals build up fluid, which is called ascites and which can be drawn off from the abdomen and contains high concentrations of the monoclonal antibodies. Either the culture fluid or ascites fluid containing the antibodies can be subjected to purification by precipitating the antibodies with protein A. The resulting relatively pure antibody preparations are quantified based on the micrograms of protein.

Polyclonal antisera, in contrast, are derived by bleeding animals a few weeks after they have been immunized. Usually several "booster immunizations" are given, and several bleeds are taken. Blood volume in a mammal is usually ~7% of body weight, and typically ~10–15% of total blood volume may be exsanguinated at any one time without injury to the animal. Hence, a single bleed from a 3-kg rabbit may be 25 ml, whereas a bleed from a 30-kg goat can be 250 ml. When the red blood cells are spun down from the clotted blood, the remaining serum is usually about one half this volume. As a result, a single bleed from a larger animal can be used for a much larger number of IHC reactions than a bleed from a smaller animal. The advantage of having the larger amount of serum per bleed is that each bleed is essentially a unique combination of antibody clones. Even when boosting the same animal with repeated immunizations with the same antigen, the antibody content in sequential bleeds may differ markedly. Hence, the lot for a polyclonal antiserum is critical, and even another batch from the same animal may have entirely different staining properties. For this reason, experienced immunohistochemists write down the lot numbers for each vial of antiserum and, when they have a good lot, buy up as much of that lot as they are likely to need in the foreseeable future to avoid inability to finish a project.

Synthetic Peptide Antigens and Antigen Mapping

The ability to create synthetic proteins and peptides has revolutionized the way in which antibodies may be made and how they can be characterized. Synthetic peptides are usually from a few amino acids up to ~25 or 30 in length. The current peptide synthesis technology results in decreasing yields as the peptide lengthens, so that synthetic peptides much longer than this, while possible, are not practical. On the other hand, much longer amino acid sequences can be prepared by recombinant technology, in which a corresponding nucleic acid sequence is expressed either in a cellular or cell-free protein expression system. It seems obvious that the exact sequence used to create the antibody is critical to its properties, and hence, we will return to this issue in the criteria for antibody suitability.

At the same time, the availability of amino acid sequences from different parts of the parent target molecule has allowed us to identify the target sites in the native molecule to which the antibody binds. When the antibody binds to a partial sequence or a partial sequence competes against binding to the native molecule, the epitope, or structural features that the antibody recognizes, is presumed to be located in that sequence. This method is used to map the epitope that the antibody binds. However, this does not indicate what the sequence was of the original immunogen, because the antibody may have been made against an overlapping sequence.

Another trick to increase antibody yield is to bind the immunogen to a supporting protein, such as BSA or keyhole limpet hemocyanin. This may increase the antigenicity, particularly for a single amino acid or short peptide. However, it is critical that the resulting antiserum be preadsorbed against the supporting protein to remove antibody clones against that target.

Antibodies Against Different Portions of the Same Molecule

A related topic is the ability to generate antibodies against synthetic peptides that are derived from different components of the same molecule. Thus, it is possible, for example, to have antibodies against a large protein target that specifically bind to the N- or the C-terminal portions of the protein. This possibility gives us a powerful potential tool to use in determining antibody specificity. When the two different antibodies stain exactly the same pattern, it is highly likely that they are staining the correct target.

Antibodies Against Phosphorylated or Glycosylated Epitopes

Another possibility provided by the use of synthetic antigens is to prepare immunogens that are specifically altered, for example, with phosphorylation, glycosylation, or some other post-translational modification. Antibodies prepared in this way may be able to distinguish between different modified forms of the same molecule with great accuracy. However, showing this specificity requires appropriate controls (such as staining after dephosphorylation).

Antigen Retrieval Methods

Another major advance has been the development of methods for reversing the effects on tissue of aldehyde fixation (Guan et al. 2008; Long and Buggs 2008). Because aldehyde fixation is a reversible chemical reaction, but requires a high activation energy, heating tissue to 95C in the presence of an acidic pH (favoring the conversion of aldehydes to organic acids) can reduce the oxidation reactions that occur during fixation. This may relieve the protein of steric hindrances or specific configurations that prevent antibodies from reaching parts of the molecule. The result may be that an antibody that stains the tissue poorly or not at all in the fixed state may gain considerable binding to the target molecule. Another use for this procedure is for tissue that is old and "overfixed." The aldehyde fixation reaction is very slow and proceeds for several years. Hence, reversing the degree of fixation may "retrieve" staining of an antigen that may otherwise be beyond recognition by the antibody.

Another type of antigen retrieval process is provided by the use of a peptidase to strip surface peptide sequences off a fixed protein, which may show epitopes that were sterically inaccessible in the fixed protein. This method has also been used to improve the staining in fixed tissue with antibodies that recognize a protein in the aqueous state.

Rules for Judging Whether an Antibody Is Showing What Is Expected in Tissue

Most investigators want to use antibodies to localize cellular components and do not want to have to become experts in immunology or IHC to do so. Hence, it is useful to have a set of criteria for what constitutes a reasonable degree of assurance that the antibody being used is actually targeting its correct antigen. The answers to the questions that follow are ones that investigators should ask for each antibody they are acquiring, before they ever use it in an experiment (why waste time on an invalid antibody?). If all investigators followed these rules, the literature would be much more accurate, and investigators would avoid wasting a lot of time on invalid antibodies.

What Immunogen Is Used to Raise the Antibody?

The first critical criterion in locating a valid antibody is that the immunogen against which the antibody was raised must be known. A key principle of science is that

the work must be repeatable. Hence, if the antibody is raised against a "proprietary" antigen (usually a secret amino acid sequence, to avoid competitors from copying the product), it simply is not valid for serious scientific work. Some manufacturers have claimed that their "intellectual property" must be protected if they are to provide antibodies in the future, but in fact, this has become a routine process, and for most antibodies there are multiple manufacturers who do provide the sequence for their antigens. More importantly, if protecting their profits interferes with science, it is the use of their product that must be eliminated. Other manufacturers have claimed that they will provide their proprietary product to other laboratories in the future, so that the result of the experiment is repeatable. However, there is tremendous turnover in this field, and companies frankly are in business to make profits and not to protect scientific integrity. If they find tomorrow morning that they can make more profit selling shoes than antibodies, that is exactly what they will do, and no one will be able to repeat the work. Hence, a key issue in buying any new antibody is to avoid products for which the identity of the immunogen is not provided at the time it is purchased.

What is the Evidence That the Antibody Binds Specifically to the Expected Target Molecule in the Tissue of Interest?

The second key criterion for using an antibody in a scientific project should be to obtain at least reasonable evidence that the antibody does bind to its expected target in the tissue in which it will be studied and not to something else. This is often provided by a Western blot, which should show that the antibody stains a single band (or a set of bands) of appropriate molecular mass for that target. Note that if extraneous bands are stained, this indicates that the antibody has other additional targets in the tissue and should raise red flags against using that antibody for IHC, unless you have taken additional precautions. For example, we have seen authors take tissue from mice in which the target protein was deleted (as shown by Western blot) and preadsorb the antiserum against tissue from the knockout mouse before using it to stain the brain. This is a lovely control that removes the extraneous staining and provides strong confidence that what is stained is the target molecule.

Note the importance of doing the Western blot in the same tissue and species as the antibody will be applied for IHC. It is quite possible for the antibody to see only one band in some tissues but to see multiple extraneous bands in other tissues from the same animal. Similarly, manufacturers often try to "prove" specificity by running the antibody against a gel preparation of purified or recombinant protein. This may show that the antibody can bind to its target but does not tell anything about what else it may bind to in tissue.

Other types of specificity studies can be done. For example, for small molecule immunogens, the antiserum may be reacted against multiple similar molecules in a dot blot or liquid phase assay (ELISA or RIA).

What Controls Can Be Done to Insure That the Antibody Binds in Fixed Tissue Only to Its Target Molecule?

Despite our best attempts to insure specificity of the antibody against native proteins in the aqueous phase, ultimately we have to apply it to fixed tissue. In the fixed state, it is possible that the antibody that works well in a Western blot will find that its target antigen is distorted by the fixation process and no longer recognizable. In fact, this occurs so often that most manufacturers mark antisera as usable for Western blotting or IHC, and the latter are by far the rarer.

When polyclonal antisera are raised against a peptide antigen, it is common that most of the antisera that are produced will stain fixed tissue poorly or not at all. In one case in which the author screened antisera, we found only 2 of 31 against a common peptide hormone that could be used to stain brain tissue. If one applies the mathematics of a Poisson distribution to this problem (i.e., assume that the probability of stimulating a single antibody clone that recognizes the fixed molecule is an independent event), it is likely that, in most polyclonal sera, the antiserum is staining the tissue with only one or at most a small number of antibody clones (i.e., that the polyclonal, which may contain thousands of clones against other antigens the host animal encountered in its lifetime, is functionally a monoclonal or oligoclonal for this purpose).

One of the best tests to show that the antibody can identify its target in fixed tissue is to transfect the DNA for the target protein into cells that normally do not make it in tissue culture. The transfected and untransfected controls can then both be fixed and stained, and the presence of staining in the transfected cells shows that the antibody really does stain its target. However, this control does not prove that the antibody will only stain its target in the tissue of interest.

Another control for specific staining in tissue is the preadsorption test. Mixing the diluted antibody with an excess of the immunogen should completely block staining. This shows that the staining in the tissue is against something that is at least cross-reactive with the original protein (although it does not prove that this is what the target in the tissue actually is). In general, when the original immunogen is readily available, such as for a synthetic peptide, the preadsorption test should be run as a matter of course. This is less practical for large protein molecules and antibodies against

partially purified tissue components. Note that the preadsorption control is meaningless for a monoclonal antibody (which is produced by screening for its binding to the target, and therefore will always bind it and always pass a preadsorption test, by definition) and for antibodies that have already been affinity purified (for the same reason).

As a practical matter, the best controls for assuring that the staining in the tissue is the target molecule involve one of two approaches (Lorincz and Nusser 2008). First, if the staining is being evaluated in mice or a closely related rodent species, and there is a strain in which the target molecule is deleted, the absence of staining is a strong confirmation of specificity. Unfortunately, this is not a perfect test, because the target that is stained in the tissue may be a related molecule that is downregulated in the knockout animal. In addition, this approach only applies to situations where there is a knockout strategy available, which limits it to a few model species. Finally, in many so-called knockout mice, the original protein is not entirely eliminated. If only a portion of that protein is still expressed, it may have no functional presence but still stain with your antibody. Hence, it is critical in a knockout control to make sure what the actual gene construct is and what is actually expressed.

The second molecular approach to confirming identity of the staining was alluded to above in the section on making antibodies against different components of the same target molecule. When the two antibodies are made in the same species, showing that the staining patterns are very similar is a strong control. When the two antibodies are made in different species, simultaneous staining and showing colocalization is an even more satisfying and persuasive control.

The methods described above are not by any means exhaustively detailed. There are many clever and innovative ways that are identified by investigators to test their antibodies each year. Science is endlessly creative, and we are always finding new methods and ways of improving older methods. At the same time, we are always uncovering new ways that nature can fool us. Thus, no antibody localization is really perfect, although following the practical guide provided here should help investigators, especially those who are new to the mysteries of IHC, to insure the scientific integrity of their work.

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The Journal of Histochemistry & Cytochemistry