Diagnostic Immunohistochemistry

What Can Go Wrong and How to Prevent It

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- Context.—There are a number of critical factors that can lead to incorrect results if the diagnostic pathologist performing immunohistochemistry is unaware of, or not vigilant about, their influence.

Objective.—To highlight 3 arenas in which errors may be introduced.

Data Sources.—For choosing the correct primary antibody, selection of the most appropriate antibodies for a given clinical application can be aided by obtaining information from the vendor; however, this can yield incomplete information. There are a number of online databases that have comparisons of antibodies from different vendors, particularly with respect to their use and properties. Reading the published literature can assist in this process, particularly with respect to determining antibody sensitivity and specificity, but it is a daunting task to keep up with all of the immunohistochemistry-related papers published. Finally, Web sites of a number of quality assurance organizations are accessible and can provide a wealth of information comparing the “real world” performance characteristics of different antibodies to the same target protein. False-positive signals can result from a number of factors, including the use of inappropriately high antibody concentration, and “pseudospecific” signal that is in the wrong compartment of the cell. False-negative signal can result from factors such as use of a nonoptimized epitope retrieval method. It is critical that epitope retrieval methods be optimized for each antibody employed in the laboratory.

Conclusions.—By paying attention to these potential problems, the “black box” of diagnostic immunohistochemistry can be made more transparent.

The great British science fiction writer, inventor, and futurist Arthur C. Clarke noted that “any sufficiently advanced technology is indistinguishable from magic.” Immunohistochemistry (IHC), a technique invented in the 1940s by Albert Coons and colleagues, is now performed, 70 years later, in thousands of laboratories around the world, many with closed autostainer systems that can resemble proverbial “black boxes.” As such, this advanced technology, may, in fact, appear to be inexplicable, especially to the neophyte. But it is important to have an understanding of the methodologies and to recognize the problems that can make the result incorrect. It is important to realize that IHC is not a “special stain,” akin to periodic acid–Schiff or trichrome stains, but is rather a slide-based test procedure that enables the detection of specific proteins, sugars, etc., in tissue sections, while simultaneously preserving tissue of histology. In the evaluation of the performance of IHC in the diagnostic pathology laboratory, and in analyzing possible problems, it is not always possible to isolate the actual IHC procedure from the other “preanalytic factors” to which the procedure is linked. Immunohistochemistry as a procedure is affected by preanalytic variables, such as tissue fixation, embedding the tissue in paraffin, and performance of epitope retrieval, as parts of the actual IHC procedure. But perhaps the largest factor determining the correct interpretation of the results is the pathologist reading the slides; despite the best education, knowledge, and experience, misinterpretations can occur. Different antibodies to the same target molecule can produce very different results, and therefore this choice is a critical one.

In this review, I will focus on 3 common problems facing the diagnostic pathologist in the use of IHC: antibody selection, false-positive IHC signals, and false-negative IHC signals.

ANTIBODY SELECTION

There are 4 major resources that can be employed to help select an addition to the laboratory’s IHC antibody library. These are the vendor, online databases, published literature, and independent quality assurance organizations.

The Vendor

There are a number of reliable vendors of immunohistochemical reagents, and it is generally a matter of experience and word-of-mouth recommendations that can help the pathologist select the best antibody vendors. Virtually all
vendors supply “product information sheets” describing the antibody, its protein (or other) target, and perhaps its clinical applications. Nonetheless, even the best vendors may supply antibodies that are incompletely or even incorrectly characterized (see below). And antibodies that are “FDA cleared” (Food and Drug Administration cleared) are not necessarily better characterized (or even better reagents) than those that have not been “FDA cleared.” Nonetheless, in cases where an FDA-cleared and a non-FDA-cleared reagent to the same target are both available, the pathologist, in some situations, must select the FDA-cleared reagent.

Online Databases

There are a number of online databases that can assist the pathologist in selecting the most appropriate antibody to target a specific molecule or marker. Some of the more important Web sites are listed in the Table. Most of these Web sites contain lists of antibodies along with tables that detail critically important information, such as the type of antibody (eg, monoclonal versus polyclonal, rabbit versus mouse) and the tested applications of the antibody (eg, IHC, Western blot, immunoprecipitation, etc). There are many antibodies that will work well as reagents for Western blots or immunoprecipitation but will not work in IHC. And there are antibodies that will work on IHC studies performed on frozen tissue sections, or cells grown in vitro, but will not work on deparaffinized, formalin-fixed tissue. In addition, the antibodies may work for IHC on deparaffinized, formalin-fixed sections, but only under conditions not necessarily determined by the vendor or listed in the Internet resources. Once received, it will be critical to optimize every new antibody introduced into the laboratory with its preferred epitope retrieval methodology, particularly with respect to pH and buffer, as noted by Shi et al.3

Published Literature

There are a number of pathology publications, including this one, that reliably, every month, detail the results of studies of IHC applications to problems in diagnostic surgical pathology. However, it is nearly impossible for the practicing pathologist to keep up with the volume of papers published even in the most popular surgical pathology journals. To make matters worse, papers employing IHC as applied, for example, to problems in oncology, are increasingly published in the nonpathology literature, making them even more difficult to follow. The fallout in missing new publications includes the potential failure to incorporate new markers with significantly increased sensitivity compared with currently existing ones, as well as the persistent use of antibodies with relatively poor specificity. However, when one does read a paper describing the IHC findings of a new antibody, my recommendation is to first read the Materials and Methods section of the paper to determine the source of the antibody and learn how well it has been characterized. Look at the photomicrographs: Is the signal in the anticipated compartment of the cell (eg, if an antibody to a nuclear transcription factor, is the signal exclusively in the nucleus, and not the cytoplasm?)? As noted below, the published IHC literature is still replete with antibodies with specificities that are not exactly what was assumed by the authors.

Independent Organizations

Often, several different clones of monoclonal (rabbit or mouse) antibodies targeting different epitopes of the same protein are available, sometimes even from the same vendor. Independent IHC quality assurance organizations, such as Nordic Immunohistochemical Quality Control (NordicQC, Aalborg, Denmark; http://www.nordiqc.com) and the Canadian Immunohistochemistry Quality Control (cIQc, Vancouver, Canada; http://www.cpqa.ca), regularly publish the results of surveys of the performance of many laboratories employing different antibodies to the same target. It is possible to review the results of these assessments and select not only antibodies but platforms and optimal procedures (heat-induced epitope retrieval, dilution, antibody incubation time).

How can different antibodies to the same target molecule behave differently in IHC assays? Even among antibodies made in the same species (eg, mouse or rabbit), there can be dramatic differences in antibody sensitivities, as well as antibody specificities (eg, cross-reactivity with nontarget proteins). Furthermore, there can be significant differences in optimal protocols required by different antibodies (eg, epitope retrieval methods).

One compelling example of striking differences in antibody sensitivities is found in a recent study in which the sensitivities of several different antibodies to the intestinal transcription factor CDX-2 were compared.4 The rabbit monoclonal antibody to CDX-2, EPR2764Y, was found to be superior in sensitivity to the other anti–CDX-2 clones tested, being the only antibody that identified the subpopulation of CDX-2–positive cells in the normal pancreas. In a different study of the comparative sensitivities of two different TTF-1 antibodies, the SPT24 rabbit monoclonal antibody was found to be significantly more sensitive for the detection of all lung carcinoma histologic subtypes than the 8G7G3/1 mouse monoclonal antibody.5 Nonetheless, several studies have demonstrated decreased specificity of the SPT24 compared with 8G7G3/1.6

The importance of knowledge of differences in antibody specificities can be highlighted by recent experience with antibodies to the gynecological and genitourinary nuclear transcription factor PAX-8. Several studies published in 2011 documented widespread expression of this transcription factor in normal tissues and in tumors of the kidney, ovary, and thyroid, where it is still employed today. However, these studies also “documented” PAX-8 expression in lymphomas and thymomas.7-9 More recent studies have shown that the rabbit polyclonal antibody employed in these 2011 studies is not completely PAX-8 specific. This polyclonal antibody apparently cross-reacts with two other members of PAX family, PAX-5 and PAX-6, a fact that was unknown when the vendor was first selling this antibody. In fact, using more specific monoclonal anti–PAX-8 antibodies, it has been demonstrated unequivocally that PAX-8 is not expressed in thymic tumors or lymphomas.10-12

Recommended Antibody Resource Web Sites

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<td><a href="http://www.proteinatlas.org">http://www.proteinatlas.org</a></td>
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<td><a href="http://www.antibodyregistry.org">http://www.antibodyregistry.org</a></td>
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of this unfortunate “mischaracterization” of PAX-8 by using antibodies of inferior specificity is that it is quite important for the pathologist performing immunohistochemistry to have reasonable certainty about the specificity of the antibodies being employed in the laboratory. In the case of the cross-reacting PAX-8 antibodies, overreliance on the information provided by the vendor was partly responsible. And although it may be beyond the scope of the diagnostic

Figure 1. Effect of increasing antibody concentration on immunostaining signal, producing false-positive signal at excess antibody concentration. A, Hematoxylin-eosin–stained section of papillary lung adenocarcinoma. B through F, Anti–PAX-8 antibody BC-12 at working dilution, 1:250 (B); 1:10 (C); 1:50 (D); 1:25 (E); and 1:10 (F). Note the appearance of false-positive nuclear (and cytoplasmic) signal at highest concentrations (original magnification ×200).
pathology IHC laboratory, there are well-defined hierarchies of proof of antibody specificity that can be tested. However, antibodies employed in the diagnostic IHC laboratory have rarely been subjected to high standards of determination of their specificity. Perhaps the highest level of proof of antibody specificity, and one that very few IHC reagents attain, is the abrogation of signal using tissues either genetically deficient for the protein, or cells that have been treated with siRNAs which completely abrogates expression of the protein in question.13

**FALSE-POSITIVE SIGNALS**

The most common causes of false-positive signals in IHC preparations include nonspecific background signal, endogenous peroxidase, the use of inappropriately high antibody concentrations, pigment mistaken for true signal, endogenous biotin, drying artifact, and “pseudospecific” signal. I will focus on 3 of these factors.

High nonspecific background generally represents binding of antibodies by mechanisms other than specific binding of their epitope on the target antigen. This signal can generally be blocked with any protein that does not have an affinity for the target or program components. Normal serum, bovine serum almond, gelatin, and dry milk are generally good blocking reagents.

The effect of inappropriately high antibody concentrations can be seen in Figure 1. Note that with increasing antibody concentration a tumor (papillary lung carcinoma) known not to express the transcription factor (PAX-8) in question can develop significant nuclear signal by just inappropriately increasing the primary antibody concentration.

A “pseudospecific” signal is one that is characterized by a signal that is usually quite restricted in distribution within a cell but actually represents a false-positive signal. Most of the commonly employed markers in diagnostic surgical pathology have well-defined subcellular localization (Figure 2). For example, with rare exceptions, nuclear transcription

![Figure 2.](image)

Importance of identification of proper subcellular compartment immunostained in identifying “pseudospecific” false-positive signal. A, Appropriate predominantly membranous signal in infiltrating breast cancer with antibodies to the transmembrane receptor HER2. B, Appropriate granular cytoplasmic signal in melanoma with antibody HMB-45, identifying the premelanosomal marker gp100. C, Appropriate nuclear signal in lung carcinoma with antibodies to the nuclear transcription factor TTF-1. D, Inappropriate membranous signal noted with antibodies to synaptophysin in lung adenocarcinoma. This antibody should produce a granular cytoplasmic signal, and this “pseudospecific” pattern clearly represents false-positive immunostaining (original magnification ×200).
factors, such as TTF-1, PAX-8, and CDX-2, are found exclusively in the nucleus. Markers such as chromogranin A and gp100 (the target of antibody HMB-45) localize to granular structures within the cell and tumor cytoplasm, in dense core granules (chromogranin A) and premelanosomes (gp100), respectively. "Pseudospecific" signals can generally be identified by the inappropriate subcellular localization of the signal. For example, a nuclear signal with antibodies to synaptophysin should easily be recognized as a false-positive signal (Figure 2, B). However, there are rare examples of "pseudospecific" false-positive signals occurring in the same cell compartment, as was shown several years ago using the SP2 anti–progesterone receptor monoclonal antibody, which also yielded a false-positive nuclear signal in progesterone receptor–negative cells.14

FALSE-NEGATIVE SIGNALS

The most common causes of false-negative immunostaining are poor tissue fixation, antibodies too dilute or not properly optimized, and epitope retrieval method not optimized for individual antibodies. As shown in a recent study from the cIQc, many laboratories have serious problems with false-negative immunostaining due to inappropriate calibration and optimization of pan-keratin and anti–low-molecular weight keratin antibodies.35

As was shown by Shi et al3 several decades ago, no single epitope retrieval solution is optimal for all antigens or antibodies, and it behooves the individual laboratory to use a “test battery” of epitope retrieval buffers and pHs in order to avoid suboptimal protocols for individual primary antibodies.3 The importance of using optimal protocols is demonstrated in Figure 3.

CONCLUSIONS

The diagnostic pathologist using IHC ignores these potential problems at his or her peril. Lack of awareness of these and other pitfalls in diagnostic IHC may lead to potentially serious errors in diagnosis, affecting patient management.

References


