



# Controls for Immunohistochemistry: The Histochemical Society's Standards of Practice for Validation of Immunohistochemical Assays

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## Summary

Immunohistochemistry is widely used in biomedical research to localize specific epitopes of molecules in cells and tissues. The validity of interpretations based on immunohistochemistry requires appropriate positive and negative controls that are often not reported in publications. This omission may lead to incorrect interpretations and irreproducible results in the literature and contribute to wasted time, effort, and resources as well as erosion of confidence in scientific investigation by the general public, legislative bodies and funding agencies. The present article summarizes essential controls required for validation of immunohistochemical findings and represents a standard of practice for the use of immunohistochemistry in research and diagnostic investigations. Adherence to the guidelines described in the present article can be cited by authors as support for the validity of interpretations of the immunohistochemistry reported in their publications. (*J Histochem Cytochem* 62:693–697, 2014)

## Keywords

Immunohistochemistry, immunocytochemistry, antibodies, controls, validation, assay, standards

## Précis

Immunohistochemistry is no different from any other technique: An experimental approach for which the quality of the results relies entirely on the analyte, reagents, precise experimental technique, correct application of controls and, ultimately, an accurate interpretation of the data based on all of the aforementioned factors. In this article, the essential nature of appropriate controls in immunohistochemical assay is discussed.

## Introduction

The *sine qua non* of validating research findings is the use of appropriate controls in the design and performance of experiments and assays. Unfortunately, in the case of immunohistochemistry, although the need for controls is well

established (Baskin 2009; Burry 2000, 2010; 2011; Frevert et al. 2013), it is the experience of the authors that critical controls are often not performed or reported in ensuing publications. This neglect has resulted in the publication of unverified and irreproducible findings in the literature (Couchman 2014; Collins and Tabak 2014). Contributing to this problem is the fact that many high-impact journals that publish immunohistochemical data do not require authors to report controls for immunohistochemistry and, what is perhaps more regrettable, the controls that are published often

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are inappropriate or misinterpreted. Realizing that controls are an essential component of experimental design in all scientific investigations, we insist that valid interpretations of immunohistochemical assays cannot be made in the absence of minimally appropriate controls. Furthermore, controls must be described and included in the ensuing publications.

Simply stated, an immunohistochemical assay that lacks controls cannot be validly interpreted. Period. It's equally important not to conclude that a molecule is present when in fact it is not, as it is to conclude that it is absent when in fact it is present. Both can lead to erroneous scientific conclusions and clinical misdiagnoses. The possibility of both errors must be considered when designing an immunohistochemical assay. It is important to understand that controls—either positive or negative—can never prove the identity—or the presence or absence—of a molecule in a tissue sample using immunohistochemical techniques. With proper controls, however, the investigator can build a convincing case for the presence or absence of a probed molecule. This is the best that can be done, but it must be done correctly to be credible and reproducible.

To get a snapshot of the “attitude” of many journals for the importance of controls in papers that incorporate immunohistochemical methods, we examined a sample of 29 articles that had immunohistochemical data in the *Journal of Histochemistry & Cytochemistry* and 71 additional publications in eight other high-impact cell biology and pathology-oriented journals that routinely publish data and images based on immunohistochemistry. Specifically, we were interested to know the extent to which control results are readily identifiable in articles that included immunohistochemistry in these journals. We also reviewed guidelines for authors of all of the selected journals. Of the 100 articles in nine journals, up to 80% of papers do not mention controls and 89% of guidelines for authors for these journals do not require or even mention controls. This informal survey sheds light on one reason that controls are often omitted from publications: journals (and, by inference, their reviewers and editors) either do not understand the basic principles of immunohistochemical controls or they do not consider them sufficiently important to require inclusion in their articles. This sadly influences investigators to have an uncritical attitude towards using valid controls in their studies.

Accordingly, we recommend that the guidelines for authors for all journals should include a requirement that controls must be described and included in manuscripts that report results of immunohistochemical studies. To avoid inaccurate conclusions on a false-positive or a false-negative result, a minimum of a positive and a negative control should be run for all immunohistochemical assays. Moreover, journals and their editors should require reviewers to comment on whether appropriate minimal positive and negative controls were used. Here, we briefly describe these controls and how they should be correctly interpreted.

## Antibody Specificity

Essential to valid interpretation of immunohistochemical staining is the selection of antibodies that have been validated to detect specific epitopes. This issue is perhaps the most critical “control” and is discussed extensively elsewhere (Gore 2013; Fervert et al 2013; Saper 2009; Saper and Sawchenko 2003). The case for specificity is often established independent of binding to epitopes in tissues or cells. This usually involves the separation of proteins that differ in size, charge, or conformation using gel electrophoresis. The proteins are then transferred from the gel to a membrane (i.e., western blot), where they are stained with an antibody and/or antibodies specific to the target protein to resolve the issue of the cross-reactivity of antibodies (Marchalant et al. 2014). Although this specificity is usually provided by the manufacturer of the antibody, ideally the proteins separated in the western blot should be obtained from the same tissue or cells as the antibody will be used for immunohistochemistry. The latter is often not feasible for an investigator but, nevertheless, the investigator must realize that this meaning of “specificity” applies only to the property of antibodies (either polyclonal or monoclonal) to recognize specific epitopes but does not prove the identity for binding of a “stained” targeted molecule in a tissue sample. Unfortunately, the so-called “absorption control” is often incorrectly offered to as a negative control as support for the specificity of immunostaining of a targeted molecule. This procedure is carried out by mixing the antibody with the target protein before applying the antibody to the tissue slice. If immunohistochemical staining in the tissue is blocked by this procedure, the staining observed with the unabsorbed antibody is often considered to be specific. However, this result only demonstrates that the antibody is specific for the molecule to which it was generated—a fact already demonstrated by western blots—but does not demonstrate that the antibody is binding to the same target molecule in the tissue. Unfortunately, in the spirit of considering the need for controls, reviewers often ask for absorption controls but this result (i.e., blocking of immunostaining) is a weak control for making conclusions about the identity of targeted molecules in the tissue (Holmseth et al. 2012).

## Positive Controls

Positive controls in immunohistochemistry protocols are specimens containing the target molecule in its known location (e.g., specific cell type, intracellular compartment, among others) and whose histomorphology and cytomorphology can be visualized by a “stain” (i.e., the fluorochrome of a chromogenic molecule). Generally, these characteristics are initially deduced from other experimental evidence, including fractional western blots, overexpression, knock-down, experiments on cells, or studies carried out on transgenic animals known to express

the target molecule. Conversely, the antibody should not produce staining in genetically engineered mice where the target molecule has been confirmed to be deleted. By and large, the use of cell lines, especially those transfected to over-express a target, are not recommended as appropriate positive controls, as many assays lack the appropriate dynamic range and calibration to ensure appropriate comparison between a cell line and tissue.

The most rigorous positive control is the positive anatomical control; i.e., where the presence of the antigen in the specimen is known a priori and is not the target of the experimental treatment. This can be a known site of expression of the target molecule in the specimen (i.e., “internal positive control”) or a separate specimen (e.g., a slide) that is known to contain the molecule targeted by the antibody (i.e., “external positive control”). For example, an assay that uses an antibody that is claimed to be specific for insulin should include sections of pancreas that have islets of Langerhans; the antibody should target and visualize only the insulin-producing islet beta cells. The antibody should have been demonstrated not to cross-react with closely related molecules of the insulin-like peptide family. Interpretation of positive controls can be problematic in the case of antibodies that are directed against molecules that are expressed ubiquitously or are expressed at very low levels, and this criterion for specificity may be impossible in clinical settings. Nevertheless, in the absence of such controls, the investigator is obligated to (and journals should demand) that authors be circumspect about interpretations derived from such assays.

## Negative Controls

The goal of a negative control is to demonstrate that the reaction visualized is due to the interaction of the epitope of the target molecule and the paratope of the antibody/affinity reagent. It is absolutely critical for investigators to be wary of claims made about the specificity of antibodies used for immunohistochemistry, especially for antibodies obtained from commercial sources. Although a manufacturer may demonstrate specificity of an antibody by western blot analysis, one cannot conclude that immunohistochemistry with the same antibody reveals the same target molecule in a tissue sample. Thus, in addition to the molecule targeted by the antibody, the results of an immunohistochemical assay (e.g., “stain”) can reveal the presence of structurally related molecules as well as the binding of the antibodies used in the assay (both primary and secondary) nonspecifically to other cellular and tissue components. Accordingly, journals should require of authors to demonstrate (or cite evidence) that the antibody recognizes a protein within the tissue of interest that migrates appropriately in the gel electrophoresis and stains with antibodies specific to the target protein, as demonstrated by a western blot.

Unfortunately, a common “negative control” seen in many articles in the literature is to perform the immunohistochemical assay with a primary antibody in tandem with a specimen that is not exposed to the primary antibody; that is to say, with the primary antibody omitted. Investigators claim—and, regrettably, journal reviewers and editors accept—this as control for the specificity of the staining for the antigen targeted by the antibody. This is a profound error. The absence of staining when the primary antibody is omitted is a control for nonspecific binding of the secondary antibody; this result is not evidence for the specificity of staining with the primary antibody. However, in some instances, it is an important additional control in assay development. It does not distinguish between staining that results from immunoglobulins binding at antigen binding sites in the F(ab)<sub>2</sub> variable regions (e.g., implicit for “specific binding” and immunoglobulins interacting with cell and tissue components in the Fc region) or due to nonspecific mechanisms. The proper negative control in this instance is substitution of serum or isotype-specific immunoglobulins at the same protein concentration as the primary antibody.

Regrettably, the use of isotype-specific immunoglobulins (or “normal” or “preimmune” serum) as a substitute for the primary antibody has fallen out of favor as a negative control for staining specificity. In the instance of a non-commercial polyclonal antibody, pre-immune sera should be used, and when pre-immune sera is not available (commercial polyclonal antibodies and all monoclonal antibodies) an isotype-specific immunoglobulin should be appropriately substituted. This control should be an absolute minimum requirement by journals for publishing studies that incorporate immunohistochemistry. It should be understood, however, that so-called “specific antibodies”, both polyclonal and monoclonal, can also bind nonspecifically due to Fc binding and other mechanisms, leading to the possibility that both specific and non-specific staining observed with such an antibody in a tissue section could not be validly distinguished without further controls (i.e., some of the staining is specific and some is non-specific). In this situation (where the primary antibody appears to show nonspecific binding), an absorption control can be useful in distinguishing specific from non-specific staining; the staining that is absent when the absorbed antibody is used (against a background of persistent, non-specific staining) is likely to indicate specific antibody binding sites.

## Standards of Practice for Immunohistochemistry

Recognizing that the omission or improper use of controls is a serious problem limiting the validity as well as the reproducibility of much published data derived from the use of immunohistochemistry, we recommend that biomedical

**Table 1.** Essential Elements Pertaining to Controls in Immunohistochemical Assays that should be Included in Peer-reviewed Manuscripts\*.

1. Documentation or reference to a western blot confirming an antibody:antigen binding to detect the target biomolecule of appropriate molecular size in a cellular lysate (not in vitro synthesized or immunogen-only).
2. A clear statement as to the nature of the immunohistochemical positive control that was performed.
3. A clear statement as to the nature of the immunohistochemical negative control that was performed.
  - A. Negative Controls should be performed with pre-immune or isotype-specific sera.
  - B. Negative Controls that omit the primary antibody only are inadequate in and of themselves.
4. The use of genetically engineered controls, as well as siRNA, with PCR confirmation can serve as positive and negative controls.
5. The use of an alternative means of controls, including “absorption controls”, are inadequate to demonstrate specificity.

\*Additional recommendations concerning immunohistochemical assays will be defined in additional articles in this series.

journals include specific requirements regarding the inclusion of controls for immunohistochemistry in their instructions for authors, as an element of describing immunohistochemical assays. The guidelines outlined in this article are the first step in defining a standard of practice for immunohistochemistry. Additional articles will outline additional points that should be addressed to ensure adequate validation and description of immunohistochemical assays. The end product of this larger effort will be a unified manuscript outlining the specification of immunohistochemical assays in the peer-reviewed literature, and the individual articles will serve as the in-depth information on each of those points.

Although reproducibility is a concern, the validity of reported results is the more serious problem. The findings with a poor antibody can generate reproducible, but invalid, conclusions if proper controls are absent. Reproducibility can become a problem when different antibodies to the same antigen produce different results and controls are lacking to sort out the validity of different findings. In Table 1, we outline the elements with reference to controls that journal editors should require to be included in submitted manuscripts that report immunohistochemical data. The results of a positive control and a negative control as well as data supporting the claimed specificity of the primary antibodies for the target molecules should be reported.

Characterization of antigen-antibody specificity should rely on confirmation of a one-protein:one-antibody relationship, or determination of the presence of shared epitopes. When shared epitopes are present, additional controls are essential. Approaches such as “absorption control” and the omission of a primary antibody should never be accepted as sole negative controls for specificity of staining in the absence of clear statements by the authors that these controls do not unequivocally demonstrate specificity of staining. The points outlined in Table 1 can be cited by authors as support for the validity of interpretations of the immunohistochemistry reported in their publications.

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