CME/SAM

Canadian Association of Pathologists–Association canadienne des pathologistes National Standards Committee/Immunohistochemistry

Best Practice Recommendations for Standardization of Immunohistochemistry Tests*

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Upon completion of this activity you will be able to:
- classify clinical immunohistochemistry tests and determine what is appropriate quality assurance/quality control level for each immunohistochemistry test.
- select appropriate positive and negative controls for immunohistochemistry tests.
- define immunohistochemistry test optimization, validation, and verification.

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Abstract

Immunohistochemical and immunocytochemical assays are highly complex diagnostic analyses used to aid in the accurate identification and biologic characterization of tissue types in neoplastic and nonneoplastic diseases. Immunohistochemical tests are applied mainly to the diagnosis of neoplasms. Some immunohistochemical tests provide information of important prognostic and predictive value in selected human neoplasms and, as such, are often critical for the appropriate and effective treatment of patients. This document provides recommendations and opinions of the Canadian Association of Pathologists–Association canadienne des pathologistes National Standards Committee/Immunohistochemistry relevant to clinical immunohistochemical terminology, classification of immunohistochemical tests based on risk assessment, and quality control and quality assurance and summarizes matters to be considered for appropriate immunohistochemical/immunocytochemical test development, performance, and interpretation in diagnostic pathology and laboratory medicine.

Immunohistochemical and immunocytochemical assays are highly complex diagnostic analyses used to aid in the accurate identification and biologic characterization of tissue types in neoplastic and nonneoplastic diseases.[1,2] Although currently applied mainly to the diagnosis of neoplasms, some of these tests provide information of important prognostic and predictive value in selected human neoplasms and, as such, are often critical for the appropriate and effective treatment of patients.[3] These assays require specialized training in the selection of the appropriate tissue fixation and processing, preparation of the immunohistochemical/immunocytochemical slides, selection of controls, pretreatment, detection systems, and reagents and extensive training in test selection and the interpretation of results. This document addresses several topics relevant to test quality and recommends standards for appropriate immunohistochemical/immunocytochemical test development, performance, and interpretation in diagnostic pathology and laboratory medicine.

With the understanding that medical knowledge and technology are constantly evolving, these recommendations comprise current principles and best practices for quality assurance (QA) in clinical immunohistochemical testing that anticipate

the need for expansion and improvement of test options. The recommendations seek to assist Canadian pathologists and clinical immunohistochemical laboratories in the development and introduction of appropriate QA procedures to: (1) promote development and implementation of standards for QA; (2) provide a standardized approach to tissue handling, test performance, and test interpretation; (3) facilitate introduction of newly developed QA frameworks and maintenance of stringent standards for test performance and interpretation for prognostic and predictive tests, the results of which are used for stratification of patients for appropriate therapies; and (4) increase public and professional confidence in the quality of immunohistochemical testing.

These recommendations for quality control (QC) and QA in clinical immunohistochemistry provide the basis for reasoned QC/QA in the clinical immunohistochemical laboratory and to ensure the accuracy of the tests and their interlaboratory reproducibility. This document addresses almost exclusively indirect immunohistochemical methods performed on formalin-fixed, paraffin-embedded (FFPE) tissues used by most practicing anatomic pathologists and hematopathologists.

This document includes recommendations on good laboratory practices designed to ensure that appropriate quality processes are considered. It does not prescribe the use of specific reagents, methods, or laboratory equipment.[4]

This document, which is inspired by previously published immunohistochemical standardization documents and articles,[5-10] aims to provide a framework for laboratory measures for clinical immunohistochemistry in Canada, which should be of help to pathologists and medical laboratory technologists and to organizations involved in the development or the implementation of laboratory QA programs for the practice of clinical immunohistochemistry.

The Canadian Association of Pathologists is neither a regulatory nor a licensing body. These standards are proposed and recommended for use as one of the tools to achieve the aforementioned objectives in the area of high-complexity laboratory testing. The best practice recommendations for standardization of immunohistochemical tests from the Canadian Association of Pathologists–Association canadienne des pathologistes (CAP-ACP), National Standards Committee/Immunohistochemistry should be viewed as a living document expected to be frequently updated and modified to follow evolving science in this rapidly developing pathology practice.

Use of Standard Terminology

The use of standard terminology will improve communication among pathologists and laboratory staff and ensure appropriate test classification, which will further determine the level of appropriate QC and QA measures that need to be implemented by clinical immunohistochemical laboratories.

Proposed Terminology

Clinical immunohistochemical laboratory.—Any diagnostic pathology laboratory using immunohistochemical tests for the purpose of diagnosing and/or characterizing human disease, the results of which will be evaluated by a pathologist and incorporated into pathology reports.

Immunohistochemical tests.—Tests that use immunoassays to produce color change colocalizing with an epitope of interest in tissue sections. Immunohistochemical tests also encompass testing on cell blocks or clot specimens prepared from cytologic and hematologic materials. While the majority of immunohistochemical tests use immunoenzymatic detection methods and in particular horseradish peroxidase and the chromogen diaminobenzidine to demonstrate a positive reaction, immunohistochemical tests also may use other methods of detection (eg, immunofluorescence, alkaline phosphatase, and others).

Immunocytochemical tests.—Tests that use immunoassays to produce color change colocalizing with an epitope of interest in cytologic smears, cytocentrifuged preparations, or monolayer preparations. Immunocytochemical tests often use alkaline phosphatase–based detection systems (with red chromogens), although peroxidase-based techniques are also commonly used. Because processing of cytologic samples is often substantially different from the processing in immunohistochemical tests, immunocytochemical tests require different QC/QA measures with an emphasis on the use of appropriate positive and negative controls prepared under the same conditions.[11-13]

Preanalytic variables of immunohistochemical tests.—Any and all steps in tissue processing, including intraoperative tissue handling and treatment (eg, prolonged ischemia, delayed fixation), type and length of fixation, decalcification, and elements of tissue handling. The preanalytic component is concluded at microtomy and the placement of the tissue section on pretreated glass slides.[6,9,14]

Analytic variables of immunohistochemical tests.—The analytic variables phase begins with the handling of the cut slides in a clinical immunohistochemical laboratory. It is completed with the coverslipping of the stained slides.[6,9,10]

Postanalytic variables of immunohistochemical tests.—Interpretation and reporting of the results, which also includes interpretation of positive and negative control results.[6,9,15]

Class I immunohistochemical/immunocytochemical tests.—Any immunohistochemical or immunocytochemical test that is interpreted in the context of histomorphologic or cytomorphologic and clinical data. This class of in vitro medical tests includes the great majority of immunohistochemical and immunocytochemical tests, most of which are used

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for determination of cell differentiation (eg, cytokeratins, vimentin, S-100, CD45). Any immunohistochemical/immunocytochemical test that is reported as a stand-alone result to a clinician for prognostic or predictive purposes is by definition class II (see the next term). The US Food and Drug Administration (FDA) classification of immunohistochemical reagents and kits uses similar terminology, which is also based on the "risk assessment" and "level of concern."[16,17] The class I immunohistochemical test is used to designate any immunohistochemical/immunocytochemical test for which results are used only by pathologists.

Class II immunohistochemical/immunocytochemical tests.—Any immunohistochemical or immunocytochemical test that is not directly confirmed by routine histopathologic or cytologic internal and external control specimens. These tests are ordinarily reported as independent diagnostic information to ordering clinicians. Claims regarding clinical usefulness associated with these data must be widely accepted and supported by valid scientific evidence. These test results are often used to determine patient management. Examples of class II tests are those intended for semiquantitative measurement of an analyte, such as hormone receptors in breast cancer. Of note, very few immunohistochemical tests are currently in clinical use as class II immunohistochemical tests. However, the same test can be designated as class I and/or class II depending on how the results of testing are interpreted. CD117 positivity in acute leukemia is an additional marker of myeloid differentiation (class I); CD117 positivity in a stromal gastrointestinal tumor may be used for the stratification of the patient for imatinib therapy (class II). Bcl-2 positivity in Bcl-6+ germinal center cells helps support a diagnosis of follicular lymphoma (class I); Bcl-2 expression in estrogen receptor (ER)+ breast carcinoma may be considered as a favorable prognostic marker (class II). ER positivity may be used in evaluation of metastatic carcinoma to suggest the possible primary site (class I), but ER positivity in breast carcinoma is often used to stratify the patients for hormonal therapy (class II). The FDA classification of immunohistochemical reagents and kits uses similar terminology, which is also based on the risk assessment and level of concern.[16,17] The class II immunohistochemical test is used to designate any immunohistochemical/immunocytochemical test for which results are used by clinicians.

Qualitative immunohistochemical tests.—Test results that are interpreted only as positive or negative. Some quantitation may be involved because a cutoff point or threshold for interpreting the result as positive is often quantitatively defined (eg, >10% reactive cells designated as a positive result). The cellular localization of the evaluated epitope or antigen must be taken into account when interpreting these tests. These tests can be optimized without reference control material by using appropriately selected positive and negative controls. However, the calibration of these tests needs to be validated and verified in-house and further by participation in external QC/QA programs.[8,18]

Quantitative immunohistochemical tests.—The test results are interpreted and reported according to an accepted scoring scheme. The tests are usually interpreted in a semiquantitative manner (eg, from 0 to 3+), but may also be subject to evaluation by image analysis or manual cell counts. It is assumed that the tests are optimized and calibrated in such a manner that the intensity of staining, percentage of positive cells, and distribution of staining proportionally reflect the levels of target antigen expression. These tests cannot be run without prior calibration against reference control material or a specified tissue equivalent. Optimally, the performance of class II quantitative immunohistochemical tests will be informed by national/international consensus guidelines that address issues relating to and performance guidelines for preanalytic, analytic, and postanalytic variables.[8,18-21]

Prognostic immunohistochemical tests.—The results of these tests independently forecast clinical outcome. They may be qualitative or quantitative.[2,22,23] They are considered class II immunohistochemical tests.

Predictive immunohistochemical tests.—The results of these tests independently predict response to a particular therapy. They may be qualitative or quantitative. They are considered class II immunohistochemical tests. These tests are currently limited to those for which targeted therapies are developed (eg, HER2/neu in breast carcinoma, CD117 in gastrointestinal stromal tumor, CD antibodies in lymphomas/leukemias).[2,22,23]

Controls.—Devices, solutions, lyophilized preparations, cell lines, or human tissues intended for use in the QC process.[1,2,6,24]

Calibration of immunohistochemical tests.—Calibration of immunohistochemical tests is based on results obtained by analysis of appropriately selected positive and negative controls or designated reference controls. Calibration of qualitative tests (class I) is based on the use of semiquantitative controls that include at least 1 tissue sample with weak expression of the target antigen and 1 sample with moderate or strong expression of the target antigen. A single tissue fragment may also be suitable if it predictably represents cells with low and high antigen expression. For details on control design, see the section "Positive Controls." Calibration of prognostic and/or predictive immunohistochemical tests (class II) is based on the use of reference, previously validated control materials (calibrated control samples, which may consist of tumor samples, histoids, matrix models, or cell lines) with predetermined and reproducible levels of target antigen expression. Samples negative for the antigen of interest should be included. Commercial and homemade calibrated control samples are acceptable if scientifically validated. Optimal calibration may or may not

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be equivalent to maximal sensitivity of the tests because some tests may intentionally be calibrated so that they do not detect very low levels of expression of certain antigens or epitopes.

Antibody optimization of immunohistochemical tests.—Antibody optimization is just one part of an analytic component, in which it will be demonstrated that a certain clone or a specific lot of the primary antibody accurately and reproducibly detects its target epitope. This is not equal to "validation" or "verification" of a clinical immunohistochemical test (see the definition for "Validation and verification of clinical immunohistochemical tests").

Primary antibody selection.—Clinical immunohistochemical tests are often named according to the antigens or epitopes they detect. This should not be equated with primary antibodies that are available for detection of the antigen or epitope of choice. Antibody reactivities may have been proposed by the commercial provider or based on initial published literature regarding the characteristics of a particular antibody, but the tissue distribution of antibody reactivity with a given clone may expand as greater experience is gained, and a wider than previously recognized distribution of the target epitope is defined or cross-reactivity with nontarget antigens is encountered. Because many clinical laboratories usually do not have the means to extensively evaluate a particular primary antibody beyond internal validation and optimization as defined elsewhere in this document, a selection of a given primary antibody should be based on mature published literature or the results of external QA/QC programs.[6]

Validation and verification of clinical immunohistochemical tests.—A *valid* assay performs as designed to detect the specified antigen. A *verified* assay detects the antigen as designed in a specified tissue or specimen type. The process of validation or verification of class I and class II immunohistochemical tests is very different.[19] Validation and verification of class I tests are usually performed by using in-house samples of positive and negative controls. Detailed knowledge of antigen and epitope distribution and levels of expression in different tissues is required for appropriate selection of controls.[1,2,6] In contrast, an ideal validation of class II immunohistochemical tests should be based on reference material from completed prospective randomized studies, although it may also be achieved if the test and reagent sets produce results on local samples that are substantially equivalent to the originally validated immunohistochemical protocol. Concordance of 95% or more for positive and negative results with reference laboratory results or other reference method (fluorescence in situ hybridization for HER2/neu) is recommended.[19,25] The number of test samples that is required for test validation is determined by power analyses based on the proposed concordance rate and known characteristics of the calculation to be used and the expected "pass rate." The same or a different cutoff point may be used for "pass" or "fail" by external quality assurance (EQA) programs that provide proficiency testing for class II tests. Concordance at this level usually parallels a κ value of 0.80 or more, or "perfect or near perfect" agreement with a reference laboratory or method.[26] This is a desirable target for tests because the results are intended to be used to determine best therapies for patients with cancer. Some EQA programs may provide test samples that also quantitatively and qualitatively support immunohistochemical test validation and verification. Participation in such programs provides appropriate support for initial and continuous revalidation of these tests.

Principles/Best Practices for QA of Clinical Immunohistochemical Testing

Scope

Internal QC/QA standards are based on the performance of daily positive and negative controls. Therefore, the scope of this section is focused on the selection and evaluation of positive and negative controls for clinical immunohistochemical tests.[1,2,6,9,10] Such controls are calibrated according to recommended standards. Most of the published literature refers to class II tests, for which specific guidelines have been published or are in preparation.[19,27] However, for all other class II tests and the great majority of class I tests, there are no similar published guidelines. A useful source to consult is the continuously updated published literature at PubMed Search (see http://www.ncbi.nlm.nih.gov/sites/entrez). Among a few others, the NordiQC organization also posts some useful specific recommendations for control tissues, antibody selection, and optimal method (see http://www.nordiqc.org/Techniques/Recommended_control_tissue.htm).

Standardization in clinical immunohistochemistry relies on consensus regarding an "optimal result" and optimal/standardized selection of positive controls.[9,15,28] Therefore, appropriate selection of positive controls is critical for the introduction and validation of immunohistochemical tests in the clinical immunohistochemical laboratory and monitoring of daily immunohistochemical runs.[1,2,29]

Positive Controls

Positive controls consist of tissue samples that contain an antigen of interest that can be detected by using primary monoclonal or polyclonal antibodies designed to bind to the selected epitope(s) in fresh, frozen, or FFPE samples. Positive controls are valid only if they are fixed and prepared in the same manner as the tissue samples that are tested in the assay.[30,31] It is not only inappropriate to use positive controls that are processed differently from tested samples, but it may be diagnostically misleading.

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Selection of Materials and Tissues for Positive Controls

Selection is based on the following:

- Positive controls for fresh air-dried (or briefly fixed in ethanol or methanol or cytologic spray fixative) cytologic preparations must be only fresh air-dried (or fixed in the same fixative) cytologic preparations derived from previously characterized patients' samples (eg, pleural fluid with metastatic melanoma) or previously characterized cell lines.[12,32]
- Positive controls for frozen tissue samples must be previously characterized frozen patients' samples or frozen cell blocks from previously characterized cell lines.
- Positive controls for FFPE tissues must be previously characterized patients' samples or FFPE cell blocks from previously characterized cell lines. The former of these two is preferred.
- The use of normal tissues with predictable antigen expression, rather than tumor samples with variable expression of antigen, is highly recommended in the selection of positive controls, although neoplastic tissues may be of considerable value, indeed even required, in selected settings (eg, anaplastic lymphoma kinase).
- Inclusion of nonexpressor cells or tissues with expected negative results in a given positive control is highly recommended. The inclusion of such tissues provides a means for detecting unintended antibody cross-reactivity to cells or cellular components and represents a "specific negative control."
- Positive controls for decalcified tissues. Positive controls for tissues fixed in acetic zinc formalin–fixed/paraffin-embedded decalcified specimens are tissues fixed in acetic zinc formalin, embedded in paraffin, and decalcified by using the same decalcifying procedure as for patients' samples. Of note, if positive controls are not decalcified, they are much more likely to produce good positive signals with methods optimized on nondecalcified samples. False-negative results are not uncommon with such practice. It continues to be a challenge for reference laboratories to perform immunohistochemical tests on tissue samples from different laboratories for which the reference laboratory may not have appropriate positive controls. This is particularly true for bone marrow specimens, for which tissue processing protocols vary widely.[33]

Positive Controls Design

Qualitative immunohistochemical tests need semiquantitative positive controls. Semiquantitative positive controls are created by inclusion of tissues that optimally show predictable high, intermediate, and low levels of expression of the tested epitope. Samples with no reactivity for the given epitope should also be included. Such controls should ideally contain tissue with low levels of expression and intermediate or high level if both of the latter are not available. Small tissue arrays with several tissue cores with various expression levels of the epitopes are recommended as best practice. Alternatively, a single tissue fragment is sufficient if it reproducibly contains such representative areas. Examples include the following: (1) Benign tonsillar tissue contains mantle zones with a low level of CD23 expression and germinal centers with follicular dendritic cells with a high level of CD23 expression. (2) Appendix contains mucosa with crypts that demonstrate graded expression of Bcl-2. (3) Liver tissue exhibits low levels of cytokeratin 8 (or low-molecular-weight cytokeratin) expression in the hepatocytes and high levels in the bile ducts.

Quantitative immunohistochemical tests (HER2, ER, progesterone receptor [PR]) need true quantitative positive controls that are calibrated according to reference material/standard. So-called reference material can be designed by using appropriately validated immunohistochemical kits or cell lines. It can also be created by using appropriately validated tumor samples. See also the definition "Validation and verification of clinical immunohistochemical tests."

Types of Positive Controls

External positive controls are previously characterized positive tissue samples or cell lines that are tested in parallel with patients' samples.[1,2,34] For immunohistochemical tests in general, one such external positive control is sufficient per run. However, for clinical immunohistochemical testing, it is recommended that the appropriate positive control be placed on a slide together with the patient material. This external control sample needs to be indelibly identified as such. Automated immunohistochemical platforms and instruments can, in some cases, have pipetting failure with random skipping of a specimen. In some cases, in which an internal control is not present or is not informative, such instrument failure cannot be detected by any other means, other than having the external positive control placed on the same slide. It is not known how often this machine failure occurs (no published data are available); however, it can be stated that "unpublished experience of reference laboratories" strongly favors this approach to positive controls. This approach is also extremely useful in the clinical setting when an "unexpected negative" result is encountered by pathologists. The presence of an external positive control on the same slide as a patient's sample will greatly decrease number of repeated tests and will demonstrate that the analytic component of the immunohistochemical testing was valid.

Internal positive controls are tissues in the patient's sample that contain the target antigen within normal tissue elements, in addition to the tissue elements to be evaluated.[1,2,8,18] Internal positive controls are very useful if the tissue studied is expected to show at least some degree of expression of targeted epitope.

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This rule can be applied only if the test tissue is expected to demonstrate target antigen expression. Even if this is the case, pathologists should consider the variation between normal and tumor expression of most antigens. Many diagnostic criteria refer to immunohistochemical test results based on the detection of low levels of antigen(s), which are used to establish proper diagnosis (eg, CD15 in Hodgkin lymphoma, cytokeratin in small cell carcinoma), that may not be demonstrated if appropriate positive controls with exact or similar low levels are not used. Such controls are not useful if the tissue used for the study shows only aberrant tissue ("tumor only").

Negative Controls

Specific Negative Controls and Negative Tissue Controls

Specific negative controls and negative tissue controls are the tissues that are known not to contain the antigen of interest. This type of negative control enables detection of unintended antibody cross-reactivity to cells or cellular components and may be a portion of a patient sample ("internal negative control"), which parallels the concept of internal positive control. Specific negative controls are used to document no reaction in cells and tissues that are known to have no expression of the tested epitope(s).[2,9] If nonspecific negative controls are negative and specific negative controls are positive, the false-positive result is due to variables associated with the primary antibody. Occasionally, polyclonal antibodies may be contaminated with other antibodies owing to impure antigen used to immunize the host animal. This problem may be detected by the use of specific negative controls. Evaluation of cells that are expected to produce negative results in the tissue section should always be performed. Such expected negative results are more consistent in benign tissues and often unknown or unpredictable in tumors. External controls, if included in the same slide, may also provide useful information if they contain expected negative tissues.

Nonspecific Negative Controls and Negative Reagent Controls

Nonspecific negative controls and negative reagent controls are characterized as follows:

- Negative reagent controls are used to confirm the specificity of the test and to assess the degree of nonspecific background staining present by omitting the primary antibody. Commonly, the primary antibody is replaced by one of the following: (1) antibody diluent, (2) same-species nonimmune immunoglobulin of the same dilution and immunoglobulin concentration, (3) an irrelevant antibody, or (4) buffer.
- Nonspecific negative controls can detect unintended background staining. The main cause of nonspecific background staining is nonimmunologic binding of the specific immune serum sample by hydrophobic, ionic, and electrostatic forces to certain sites within tissue sections.[7,9] This form of background staining is usually uniform. Prolonged fixation in formalin or other aldehyde-based fixatives should be avoided because it may produce nonspecific background. This background staining from overfixation can be remedied by postfixation with Bouin, Zenker, or B-5 fixative, but this is not useful in daily practice.[7] Endogenous peroxidase activity is found in many tissues and can be detected by reacting fixed tissue sections with diaminobenzidine substrate, which is routinely eliminated by pretreatment of the tissue section with hydrogen peroxide before incubation of the primary antibody.[1,2,35]
- Nonspecific negative controls are sections prepared from the same block of patient material that is used for clinical testing.
- The purpose of negative reagent controls is to detect the lack of specificity of the test or nonspecific background staining and, for this reason, has been referred to as the "methodology control."
- Negative results in negative controls represent medical evidence that staining for a particular epitope in the test tissue is not a false-positive result due to variables other than the primary antibody. Therefore, negative controls are critical for daily QC/QA documentation in clinical immunohistochemistry. The following principles should be adhered to:
  (1) Negative tissue controls need to be processed in the same manner as the slides for specific immunohistochemical tests, including various epitope retrieval procedures.
  (2) The number of negative reagent controls is determined by the number of different pretreatment procedures: one negative reagent control should be prepared for each methodological variation used in a given clinical case. For example, if 3 different epitope retrieval procedures are used (eg, heat induced epitope retrieval [HIER] in citrate buffer, HIER in EDTA, and protease digestion), 3 negative controls (1 processed by HIER in citrate buffer, 1 with HIER in EDTA, and 1 with protease digestion) must be prepared.
  (3) Negative controls are typically run by omission of the specific primary antibody in the protocol and replacement by an appropriate (presumably) nonreactive moiety. For monoclonal primary antibodies, the optimal choice is an antibody of the same isotype, present in the same immunoglobulin concentration as the test primary antibody, using the same diluent, but nonreactive with human epitopes. Specially prepared commercially available negative controls may be used. For polyclonal antibodies, negative

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reagent controls should be a dilution of immunoglobulin fractions of whole serum of normal/nonimmune serum of the same animal source. Mouse ascites fluid can also be used as a negative control. Finally, the primary antibody may be replaced with cell culture medium. (McCoy tissue culture medium is commonly used.) For clinical immunohistochemical laboratories in which a large number of different monoclonal antibodies are in use, the most practical solution may be to use cell culture medium instead of primary antibody.

(4) It is important to note that none of the nonspecific negative controls are able to detect an undesirable or unexpected cross-reactivity of the primary antibody with some epitopes (see "Specific Negative Controls and Negative Tissue Controls"). Negative results with negative controls do not ensure the specificity of the immunohistochemical tests in all cases. Therefore, when unexpected reactivity of the primary antibody is encountered, false-positive results need to be considered.

(5) *Important*: When 2 or more antibodies are applied to serial sections, which is often the case in clinical laboratory, negative stain areas of one slide represent the negative control for other antibodies. This combined approach is recommended.

Exceptions

For class I tests, when a panel of antibodies is used for tissue analysis, the results of other tests in the panel may provide sufficient negative control information so that no additional negative controls are needed. This approach involves proactive interpretation of "negative control" results by the pathologist who orders the panel. When ordered, panels may not be sufficient or appropriate to serve as negative controls, and, therefore, additional negative controls could be ordered by the pathologists.

For class II tests, if published guidelines address the type of negative controls to be used for the particular class II tests, the guidelines should be followed. If negative controls are not described by published guidelines, the preceding suggested principles 1 through 4 should be followed.

Documentation of Positive and Negative Control Results

In establishing retention requirements, care should be taken to comply with provincial and federal regulations because these may exceed the following CAP-ACP recommendations.

The retention of laboratory documentation should be maintained in such manner that it demonstrates that the test(s) was performed correctly on the correct patient, that the reagents and equipment used to perform the test(s) were operating correctly, and that pathologists were given correct information, which would allow correct interpretation of the immunohistochemical results. This information is largely included in the laboratory QA/QC records. The recommendation on retention of laboratory documentation on positive and negative controls in immunohistochemical laboratories is addressed here only from the aspect of internal audit or review and its use for troubleshooting and does not address requirements for accreditation or legal purposes, which may vary in different jurisdictions and provinces. Recommendations are as follows:

- Every clinical immunohistochemical laboratory needs to keep daily records of all positive and negative controls and their results. Review and sign off of the positive and negative control results by pathologists in charge is recommended. A practical approach to recording and retention of control results is to document specific suboptimal and inadequate results. Good results can be reported in aggregate.
- If positive or negative controls indicate a failed run, documentation of corrective action is required.
- Electronic documents with secure backup are recommended.
- A 2-year period is generally recommended for the retention of all laboratory records, including the records of positive and negative control performance. However, shorter periods for retention are also acceptable as follows:
  - (1) Class I immunohistochemical test records may be kept for only 6 months. The last 10 results should be readily available for review.
  - (2) Class II immunohistochemical test records should be available for at least 12 months. The last 10 results should be readily available for review.
  - (3) If the laboratory regularly participates in EQA programs that support test validation, the records should be kept for the period of the last 2 challenges for class I and class II tests.
- It is recommended that pathologists include the results of positive and negative controls for all class II immunohistochemical tests in the pathology reports. This is not currently recommended for class I tests, although it is entirely relevant to include this information with all test results.
- Reporting of the results of the class I immunohistochemical controls is optional in pathology reports; however, appropriate documentation needs to be performed by the immunohistochemical clinical laboratory, as described.

New Test Validation

The introduction to clinical use of any new test must start with test verification and validation.[1,2,6] See "Proposed

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Terminology" for definitions. However, all previously validated immunohistochemical assays must be completely revalidated if significant changes are made to the assay procedure. Significant changes include change of primary antibody clone or provider, new detection system, new buffer type or any other component of the antigen-retrieval step, new machine for immunohistochemical analysis, or a switch from prediluted to in-house diluted (or the other way around) primary antibody. For new primary antibody lots, a smaller concordance study for lot variation alone is recommended to include a 10-case run in parallel with the original lot. Revalidation of class II tests should be performed according to published guidelines, if available.[19,25]

Class II Immunohistochemical Test Principles/ Best Practices

Scope

There is only 1 immunohistochemical test so far for which testing guidelines have been issued, namely HER2.[19] In Canada, 11 expert pathologists issued a document entitled "Canadian Consensus for HER2 Testing Guidelines in Breast Cancer," which basically reflects the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines; however, Canadian modifications are introduced based on the experience of the 11 Consensus Group Participants.[25] It details preanalytic, analytic, and postanalytic steps for HER2 immunohistochemical testing. The impact of the preanalytic component was the most challenging to define because various epitopes may have widely different responses to formalin fixation and tissue embedding or other steps in tissue processing. The following recommendations for class II tests include most recent published articles[19,25,27] and seem to be safe for all class II tests (prognostic and predictive immunohistochemical markers).

Preanalytic Component/Tissue Processing

Because ER/PR and HER2 tests are generally performed on the same specimens, at a minimum, compliance with HER2 guidelines may be sufficient for most analytic variables. However, more recently published requirements for ER/PR testing may be more informative with respect to appropriate minimum fixation times.[27] The following recommendations are based on published peer-reviewed recommendations for tissue fixation and processing of samples for breast carcinoma markers. At this time, there is no evidence to suggest that the proposed recommendations would not be safely applied to all other class II tests and to all class I tests. The following recommendations incorporate accumulated scientific knowledge on formalin-fixation effects on epitope preservation:

- Tissue processing, in particular the type of the fixative used and the fixation time before loading the tissue onto the tissue processor, needs to be recorded and included in the pathology report. Use of decalcified tissues is not recommended unless permitted by the specific class II test guidelines. If decalcified tissues are used for testing, the following times need to be recorded:
  (1) Fixation time before decalcification
  (2) Type of decalcifying reagent
  (3) Time of decalcification
- Fixation of tissues should be performed in 10% neutral pH (pH 7.2-7.6 aqueous), phosphate-buffered formalin for a minimum of 8 hours (24-72 hours optimal). A fixation time of 8 to 72 hours is generally recommended. Therefore, tissues should be fixed in formalin for at least 8 hours before being loaded onto the tissue processor.
- Non–formalin-based fixatives and/or other fixation methods should not be used for class II immunohistochemical tests because of the lack of published evidence on the performance characteristics of other tissue-processing methods. However, if any scientifically supported tissue processing method is validated in the future, such a method may be used for clinical immunohistochemical testing. It is of high importance to remember that any such preanalytic methods must also be validated for all other immunohistochemical tests (class I and class II) that may be applied to tissues thus prepared.
- The time from surgical excision of the specimen to placement in fixative should be optimized. Epitope degradation due to delayed fixation is a potentially serious problem that generally cannot be modified or corrected by epitope retrieval techniques. (These mainly adjust for fixative-induced modifications of the epitope, but not for the effects of delayed fixation, which usually leads to irreversible tissue deterioration.) Prolonged prefixation ischemic time and fixation of less than 8 hours may irreversibly modify our ability to detect specific epitopes. Samples should be sliced immediately at 5- to 10-mm intervals after appropriate gross inspection and margin designation and then placed in a sufficient volume (20:1) of 10% aqueous neutral buffered formalin.
- A longer fixation time of up to 10 days is acceptable and is not an exclusion criterion for most immunohistochemical testing, as long as the specimen has been adequately sectioned to allow adequate fixation as described. Class II immunohistochemical tests may require validation (consult published guidelines for each test separately) of protocols for fixation times exceeding 72 hours.

Tissue Processing Notes

- Underfixation is more deleterious than overfixation.
- Fixation requirements are particularly specified for ER,

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PR, and HER2 immunohistochemical and fluorescence in situ hybridization testing on core biopsy and surgical excision specimens. Consensus guidelines for other class II tests are not available yet and, thus, are not specified in this document. They will be incorporated into this document as they become available.
- Long-term archival storage of tissue blocks (for ≥20 years) does not preclude HER2 testing as long as the archived material has not been subject to significant temperature fluctuations over time.

Analytic Component

Assay validation and verification, antigen retrieval, selection of positive controls, and use of laboratory methods are according to Goldstein et al.[10] Published guidelines for class II tests should be followed.[19,25,27]

Assay Validation

As many as 50 to 100 samples may be required when validating a new antibody for a class II test. An assay accuracy of a 95% concordance rate is recommended for test validation for positive and negative categories. Ensure adequate validation, preferably by using 50% cases that are unequivocally positive and 50% cases that are the mixture of weakly positive and unequivocally negative. A much smaller number of samples may be sufficient for some class II tests (eg, CD117). Validation documentation must be kept as long as reported results of these tests are kept. Any significant modifications to the procedure require additional validation to ensure accurate performance (see revalidation for definitions).

Type of Antigen Retrieval

Stringent compliance with validated standard operating procedures developed in assay validation is required. QC documentation must be in place indefinitely or for as long as pathology reports that include the immunohistochemical test results are mandated to be retained.

Use of Standardized Control Materials

The controls should include positive and negative cases and low-protein-expressor cases. The control tissue should be fixed and processed in the same manner as the patient samples. The number of samples is determined based on the design of the validation sample and on the power analysis based on the selected level of performance and known characteristics of the test sample. It is recommended that the design of the in-house validation sample be supported by recommendations from a statistician versed in such studies. The material is validated in a prospective clinical trial or by using the results of procedures that are validated based on such prospective clinical trials.

Use of Automated Laboratory Methods

The use of correctly operated automated staining protocols and equipment is acceptable and desired for clinical testing; however, validated methods must be used. Records of recommended maintenance and the service records must be retained indefinitely or as long as the pathology reports that include results of the immunohistochemical tests are retained.

Postanalytic Component/Interpretation of Results

Every test may ultimately have its own guidelines for performance and interpretation, although guidelines for class II tests will likely be more rigidly defined than those for class I tests. HER2/neu is detailed in the "Canadian Consensus for HER2/neu Testing Guidelines in Breast Cancer" and the ASCO/CAP document.[19,25] Similar guidelines for the interpretation of ER and PR tests are being or have been developed.[27]

Image analysis may also be suitable for the interpretation of results because it was reported to be as good as expert pathologist scoring, and some systems are already approved for such use by the FDA.[36] It is recommended that image analysis scoring results also be validated by participation in an extralaboratory QC/QA program or by a reference laboratory.

The postanalytic components of class I tests are generally test-specific. However, it could be said that for general purposes, a cutoff value of 10% positive cells is used to designate a test as positive or negative. Only exceptionally, the test may be positive in smaller numbers of cells if the pattern of staining is sufficiently restricted to certain lesions and documented by the published literature. This is well illustrated by using an example of cytomegalovirus immunoreactivity in morphologically altered cells in which even 1 positive cell is sufficient for interpretation as a "positive test result." On the other hand, some class I markers are considered as positive only if strongly expressed by a majority of cells because this is the case in interpretation of some tests, most notably terminal deoxynucleotidyl transferase and CD99. Therefore, approved training in pathology is required for clinical application and interpretation of immunohistochemical test results.

Proficiency Testing: Monitoring the Quality of Laboratory Performance

Proficiency Testing and Certification of Class II Immunohistochemical Tests vs Laboratory Accreditation

Clinical Immunohistochemical Test Certification

Laboratory accreditation is currently under the jurisdiction of provincial regulatory bodies in several provinces in Canada. However, class II test certification is distinct from the current processes for laboratory accreditation. The

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immunohistochemical test certification is defined as successful participation in an EQA program for immunohistochemical testing at the pass rate of a minimum of 90% with both positive and negative results of the reference value used by the EQA program. This definition assumes the requirement of a sufficient number of test samples for meaningful statistical analysis based on power analysis. This may be accomplished by using a tissue microarray design in EQA programs.[37] It appears that at least 40 samples are required to achieve desirable pass rates (90%-95%) or to approach good correlation with near perfect agreement based on κ values of 0.80.[26] This pass rate may or may not be required by the provincial or other accrediting bodies. Canadian provinces or territories that do not have accrediting bodies may use the aforementioned recommendation for safe clinical practice. Therefore, participation in EQA programs that provide such test samples would support "certification" of clinical immunohistochemical tests. Laboratory accreditation further mandates or enforces compliance with designated provincial standards, which may be higher or lower than the aforementioned recommendation.

The preceding recommended requirements for class II immunohistochemical test certification may be regularly updated to follow published literature and developing national and international guidelines.

Certification for performing class I immunohistochemical tests would continue to be conducted according to current practices (in aggregate) with recommendation to use the Canadian immunohistochemical QA checklist for class I immunohistochemical tests.

The CAP-ACP National Standards Committee/Immunohistochemistry suggests that implementation of the national and international published guidelines for class II tests and their continuous validation could be facilitated by development of the following:

- Establishment of a Canadian national checklist for clinical immunohistochemical laboratory certification. This would ensure that all Canadian clinical immunohistochemical laboratories fulfill minimum, standard, requirements and elements of implementation for class I and class II tests, which are very different and need to be adequate for the type of tests that are performed by clinical laboratories. Class I immunohistochemical test and class II immunohistochemical test checklists would form the basis for a step-wise approach to appropriate daily QA/QC measures and for selection of appropriate EQA programs that would support test validation and verification.
- Certification for each prognostic and predictive test (class II immunohistochemical tests) separately is proposed. Historical evidence in EQA has indicated that good or even optimal performance in one test does not guarantee equally good performance in another (similar or otherwise) immunohistochemical test. Therefore, the CAP-ACP National Standards Committee/Immunohistochemistry recommends certification of each class II test separately.
- The certification of class II tests includes demonstration of at least 90% concordance for positive and negative results at least twice annually; the participation interval is aligned with ASCO/CAP recommendations for immunohistochemical breast cancer markers. Large testing centers do not automatically qualify as reference laboratories. Any immunohistochemical laboratory that provides evidence of 95% or better concordance or a κ value of more than 0.80 with both positive and negative reference values can be considered a reference laboratory because this cutoff point correlates with near perfect agreement with reference value, which is not the result of chance agreement (see preceding text regarding κ values in EQA; also, see earlier definitions of immunohistochemical test validation).
- If a laboratory did not pass with a recommended score of at least 90% concordance, it is recommended that the unsuccessful laboratory send all samples to be tested to another certified laboratory or designated reference laboratory until corrective action has been taken and repeated tests passed with an acceptable success rate. Participation in any EQA program that provides such samples for test validation is acceptable. Participation in EQA programs that do not provide a sufficient number of tissue samples may be informative and useful, but owing to the lack of an opportunity to calculate concordance rates, it does not provide sufficient information for class II test certification.
- The participation in EQA programs needs to be documented by clinical immunohistochemical laboratories, and, if suboptimal results are achieved, corrective actions also need to be documented.
- All pathologists interpreting class II tests are expected to provide interpretation that agrees with reference interpretations with 90% concordance or a κ value of 0.80 in at least 1 challenge per year.

Education and Training Standards for Laboratory Personnel

Scope

Minimum standards for clinical immunohistochemical staff including laboratory directors are not currently set. The CAP-ACP National Standards Committee/Immunohistochemistry at this time recommends that the following would be considered good practice:

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1. Clinical immunohistochemical laboratories should be adequately staffed to meet the volume and complexity of the laboratory testing.
2. Clinical immunohistochemical laboratories should be directed by a fully trained and certified designated pathologist with experience in immunohistochemistry, who is the director of the immunohistochemical laboratory and is responsible for complying with internationally and/or nationally or provincially designated standards for class II tests.
3. The director of the immunohistochemical laboratory and the laboratory manager and, ultimately, the director or head of the department of pathology in that institution are responsible for the results of the immunohistochemical tests. (It is reiterated that the director of the immunohistochemical laboratory must have final authority over the immunohistochemical laboratory and that the head of the department of pathology/laboratories must have overall responsibility for the work and results of that department.)
4. The medical director (pathologist) must have the final authority for selecting appropriate tests, antibody clones, detection systems, and any other significant component of clinical immunohistochemical testing.
5. The immunohistochemical laboratory director (pathologist) is expected to recommend the implementation of appropriate preanalytic, analytic, and postanalytic components of immunohistochemical testing. Such recommendations should be recorded. The immunohistochemical laboratory director cannot be held responsible for laboratory errors if the aforementioned recommendations are not implemented by the laboratory and hospital management.
6. Any clinical immunohistochemical laboratory should have at least 1 fully trained and qualified technologist for the type of the tests that are performed by the clinical laboratory. Support technicians and technologists without special training in immunohistochemical techniques may support immunohistochemical laboratory operations (eg, cutting tissue sections).
7. The training and continuing professional development of technologists for immunohistochemical laboratories has to be documented by department management.
8. Rotation of technologists designated to the clinical immunohistochemical laboratory to oversee and perform clinical immunohistochemical tests in addition to other histology duties is appropriate if highly qualified personnel are not replaced by less-experienced technicians or technologists without special training in immunohistochemical techniques.
9. There are no recommendations for support technicians and technologists at this time; they may rotate between immunohistochemical duties and other laboratory activities.

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