Recommendations for Improved Standardization of Immunohistochemistry

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Abstract: Immunohistochemistry (IHC) continues to suffer from variable consistency, poor reproducibility, quality assurance disparities, and the lack of standardization resulting in poor concordance, validation, and verification. This document lists the recommendations made by the Ad-Hoc Committee on Immunohistochemistry Standardization to address these deficiencies. Contributing factors were established to be underfixation and irregular fixation, use of nonformalin fixatives and ancillary fixation procedures divested from a deep and full understanding of the IHC assay parameters, minimal or absent IHC assay optimization and validation procedures, and lack of a standard system of interpretation and reporting. Definitions and detailed guidelines pertaining to these areas are provided.

Key Words: immunohistochemistry, pathology, assay, oncology, standardization, procedure, tissue, fixation

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Immunohistochemistry (IHC) is widely used in surgical pathology and serves as a diagnostic, prognostic, and predictive tool. It was recognized over a decade ago that IHC assay standardization was vital for reproducible and reliable results. Agencies, including the Biologic Stain Commission, CLSI (previously NACCLS), FDA, and the manufacturing sector established guidelines, standards, and recommendations for reagents and package inserts. These efforts have resulted in consistent, high-quality assay components and instruments on which contemporary IHC is performed.[1–4] It has also allowed the development and use of so-called black box IHC stainers in which IHC assays have preset parameters set by the manufacturer.[5]

Despite the improvements of reagents and automation, authors over the years have consistently noted the inconsistent quality of IHC assays.[6–11] Unlike previous IHC-epochs, most of the causative responsibility rests with the individual laboratory performing the IHC and specifically, the lack of standardization and attention to quality assurance programs.[12,13] Prior consensus conferences identified the likely causative factors (Table 1).[14] Recent studies strongly suggest that these problems are widespread and are not insignificant.[15–17] A 2-day *ad-hoc* consensus conference was convened in August 2006, charged with the goal of making practical and feasible recommendations directed at standardizing these laboratory and pathologist factors. The recommendations listed below are the product of this meeting that included input from the directors of IHC laboratories from a broad range of clinical practices and representing the governmental, private, and academic sectors. These guidelines do not diverge from general practice recommendations and are supported by scientific data. Unfortunately, laboratories often do not appreciate the negative impact on their specimens and the validity of IHC performed on them created by diverging away from these recommendations.

PREANALYTIC FACTORS

Fixation

Recommendation 1

Tissue should be fixed in 10% neutral-pH, phosphate-buffered formalin for a minimum of 8 hours. If formalin or formalin-alcohol mixture is a component solution on the tissue processor instrument, tissue should be fixed in formalin for 6 to 12 hours before being loaded

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TABLE 1. IHC Assay Total Test Concept

Preanalytic
Test selection
Specimen type, acquisition, transport time
Fixation: type and time
Tissue processing, type, and temperature
Analytic
AR procedure
Protocol, control selection
Regent validation
Technician training/certification
Laboratory certification
Postanalytic
Control evaluation
Results interpretation
Results reporting
Pathologist, experience and CME

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onto the tissue processor. Non–formalin-based fixatives and or alternative fixation methodologies are strongly discouraged in regard to IHC, in large part because performance data are limited and extrapolation from formalin-fixed data is unreliable.

Comments

Formalin is aqueous, completely dissolved formaldehyde. The diffusion coefficient of formaldehyde is 0.79, meaning it permeates tissue at approximately 1 mm/h.[18,19] However, permeation is not fixation. Formaldehyde becomes available after disassociating from methylene glycol in solution at a rate that is measured in hours, referred to as a "clock" reaction.[19–21] Although complete tissue fixation requires 24 hours, a minimum of 6 preprocessor hours of formalin fixation is required for consistent IHC assay results.[19,22,23]

Nonformalin fixatives and or alternative fixation methodologies are strongly discouraged for several reasons. First, tissue fixed in formalin for less than 8 hours is countered by relatively greater alcohol dehydration fixation. Although tissues fixed in this manner can produce hematoxylin and eosin-stained slides that are similar in appearance to formalin-fixed tissues, the 2 specimens have discrete differences in both histomorphology and staining characteristics by IHC. Many alcohol-fixed tissue antigens do not require antigen retrieval (AR) to be sufficiently immunoreactive and in general, the less formalin fixation, the less AR required.[24] Tissues fixed in formalin for over 8 hours requires standard amounts of AR. Five to eight hours of formalin fixation is the transition-zone between no-AR and complete-AR, and varies depending on the antigen, antibody, and tissue. The majority of suboptimal and inconsistent IHC stains result from the mixing of specimens with different AR requirements. Some antigens, especially small secreted peptides are difficult to stain and localize in alcohol or alcohol-phase–fixed tissue, as a result of a lack of cross linking and diffusion throughout the tissue.

Second, a collective body of knowledge of expected or characteristic immunoreactivity of neoplasms and lesions has accrued over time consisting of the accumulated weight of numerous published studies and decades of pathologists' personal experience. It functions as the vital foundation on which pathologists make interpretative decisions and plays a significant role in the validation of an antibody. This knowledge base is almost entirely based on IHC assays performed on antigen-retrieved sections of formalin-fixed paraffin-embedded tissue. Tissues fixed in nonformalin fixatives and or alternative fixation methodologies can produce high-quality appearing IHC slides.[25,26] However, a similar appearance is not similar antigen expression. Neoplastic and lesional tissues fixed in an alternative nonformalin method may not produce the same patterns of antigen immunoreactivity patterns as formalin-fixed tissues.[27] Some microwave processors require using the manufacturer's proprietary (and undisclosed) reagents.[28] Although similar results have been obtained in tissue microarrays, there have been no direct comparisons of antibody panel results of formalin and non–formalin-fixed standard tissue blocks, needle core, or endoscopic biopsy tissue specimens.[29] Microwave fixation induces inhomogeneous tissue fixation reactions that varies with the size specimen and composition of tissue types.[30–33] It is a flawed assumption to think that the patterns and shades of intensities in neoplasms are the same in formalin and non–formalin-fixed tissues. Demonstration of differences in the clinical significance between alternative-fixed and formalin-fixed tissues may not be readily apparent, however side by side comparisons of statistically significant numbers of specimens, usually requiring at least 30 specimens per variable are required to demonstrate equivalency. Given the increasing role IHC assays play as predictive markers of oncologic chemotherapeutic agent response, faster turnaround times and laboratory cost-containment should not be prioritized as goals ahead of the laboratory procedures required for reliable, accurate, and reproducible IHC assays.[34–37]

Third, most antibodies used by laboratories in IHC assays are classified as analyte specific reagents (ASR) and regulated as a class 1 in vitro diagnostic (IVD) device by the FDA.[38–40] The Clinical Laboratory Improvement Amendments Act stipulates that the laboratory that uses the ASR in its in-house IHC assay takes responsibility to understand, *verify*, and *validate* its performance characteristics in lesions fixed and processed in their laboratory. It seems obvious to state that the Act means laboratories are required to validate and verify that lesions fixed in their laboratory express a characteristic immunophenotype using IHC assays developed in their laboratory. Validation means that the assay performs as designed to detect the specified antigen. Verification means the assay detects the antigen, as the assay was design-based on the specimen being analyzed. For many markers, verification requires a small number of samples processed in a consistent and identical manner to the test sample to demonstrate true positive and negative staining patterns.

However, as the complexity of the IHC assay increases, especially to prognostic and predictive markers, verification of the IHC assay requires a significantly larger number of samples.

Fourth, class 2 or 3 "kits or systems" for in IVD use requires the laboratory that uses the kit or system to perform the assay in the precise and exact manner designated by the manufacturer. Examples of such kits include the HER2 HercepTest (Dako Co, Carpenteria, CA,) and Pathway (Ventana Co, Tucson, AZ). Class 2 or 3 kits or systems IVDs stipulate the permitted fixatives on which the assay can be applied, which is almost always formalin. Results of these kits or system IVDs are invalid and deemed inaccurate and potentially unreliable when they are applied to tissues fixed in method or solution not stipulated as allowable by the manufacturer.

Recommendation 2

The time and type of preprocessor fixative and the time, type, and component solutions of the tissue processor should be recorded for every IHC tissue specimen. Delineation of neutral-buffered formalin alone is inadequate; rather, specification of the type of buffer and its molar concentration is required. The same is true for reagents on the tissue processing instrument, with special attention to xylene alternatives and type of embedding paraffin. This information should accompany all tissue that is sent to an outside laboratory where the IHC staining is performed. When IHC studies are performed in a reference laboratory, the originating laboratory is responsible for recording this information for each case. The reference laboratory that performs the IHC staining should record this information for each case. This information should be permanently maintained as part of the daily work run logs of the IHC laboratory. This information should be checked against the quality of the IHC stains and be incorporated into the IHC stain quality review log performed the laboratory director or their designate.

Comment

These recommendations are in step with the NCCLS (CLSI) guidelines for HER2 IHC assays that the type of fixative and length of fixation be included in the pathology report.[41,42] Insufficient, inconsistent, and different types of fixation and tissue processing are the key factors behind suboptimal and inconsistent IHC stains on automated IHC staining instruments. In general, underfixation is a substantially larger problem than overfixation.[43,44] This recommendation plays a key role in standardizing fixation parameters for IHC assays. Ideally, the time tissue is placed into fixative would be known, in reality, this information would be difficult to obtain for most laboratories. An acceptable surrogate of fixation-start time is the time that the specimen was accessioned into the laboratory computer system or when logged into the department, providing the specimens are immersed in formalin at or before this time point. The start time of the tissue processor instrument on which the tissue block is loaded is an acceptable end-fixation time point. If tissues are fixed in formalin or formalin-alcohol solutions for an additional time period on the tissue processor, this additional length of time should be added to the total fixation time of the specimen. It is widely assumed that tissue processing is a black box process and that differences or variations in this process have little impact on the specimen. This is not true. Current evidence suggests that *any* changes in the processing system have the capacity to alter the chemical makeup of the tissue specimen. Alternative processing systems, especially those that include alterative reagents and microwaves have not been adequately validated. IHC can be performed on tissue from these systems; however, there are no well-validated studies to demonstrate equivalency.

AR

Recommendation 3

AR is presumed to "restore" the antigenicity after the formalin fixation.[45–50] The parameters of an AR protocol must be balanced to match the unique length and type of tissue fixation of the individual laboratory and the characteristics of the individual antibody.[51–53] For tissues fixed in formalin for at least 6 hours before being loaded onto a tissue processor, one AR protocol is usually adequate. Different types of AR, such as low pH buffers, high pH buffers, various types of heating devices, enzyme digestion, etc, should be available for each AR protocol, depending on the optimization parameters of individual antibodies (see Optimization and Validation section below). Although enzyme digestion is not generally considered as a component of AR, it functions as an alternative method for practical purposes. It may be the preferred method of tissue pretreatment for some antibodies.

Comment

For most contemporary oncologic antigens, a minimum threshold level amount of energy must be applied via AR procedure to all types of tissue specimens, regardless of the length of time above 6 hours the tissue specimen has been fixed.[54,55] The method of applying the energy (pressure cooker, microwave, waterbath) is substantially less important than the amount of energy applied to the slides by the AR system.[56] AR energy above this threshold generally does not improve antigen detection.[57] Poor or inconsistently optimized AR relative to tissue fixation is responsible for most suboptimal contemporary automated IHC assay results. This creates a problem of inadequate (either over or under) AR. Over-AR relative to the amount of formalin fixation produces high-background staining, section fall-off, tissue section holes and rents, indistinct nuclear detail, muddy chromatin, pseudo-nucleolar staining, and overly strong edge staining of small biopsy specimens. This type of imbalance most commonly occurs when an AR protocol intended for tissues fixed in formalin for at least 8 hours is applied to tissues fixed in formalin less than 6 hours.

Under-AR relative to the amount of formalin fixation produces false negative immunoreactivity. This type of imbalance most commonly occurs when an AR protocol intended for tissues fixed in formalin for 4 to 8 hours is applied to tissues fixed for more than 16 hours (overnight fixation) (unpublished data).

It is very difficult to optimize AR on poorly fixed and processed tissues. Adequate fixation is key to achieving reproducible and optimal AR. It is not uncommon for laboratories to encounter a mixture of under and overfixed tissues, resulting in a large spectrum of problems, which combine together to produce result in inconsistent and unreliable IHC assays. AR is a part of clinical IHC, and the best solution is to standardize fixation in an effort to obtain consistent IHC.

Analytic Factors

Proper analysis of IHC assays is critical for correct tumor identification and prognostic/predictive assessment. Pathologists who have documented expertise in this field should interpret IHC assays. Expertise is attained by documenting experience with literature use and proficiency testing. IHC is an adjunct to pathologic interpretation, and all IHC assays should always be interpreted within the context of morphology.

DEVELOPING NEW ASR ANTIBODIES INTO IHC ASSAYS

Developing new ASR antibodies into clinical IHC assays is a 2-step process of optimization and validation. Optimization is the process in which the laboratory serially tests and modifies component procedures with the end point of producing a consistent high-quality assay. Antibody optimization must be completed before moving on to the validation step. Antibody validation is the process whereby the parameters of the IHC assay, including its accuracy, reliability, and reproducibility are established.

Antibody Optimization

Recommendation 4

Reagent package inserts should be read completely and in detail before beginning the optimization process. The laboratory director should sign the package insert and it should be maintained in an easily accessible laboratory manual as a reference source during the active life of the antibody or reagent. The manufacturer recommendations listed in the package insert should be followed (Table 2).

Comments

Work by the Biologic Stain Commission, FDA regulations, and AR pretreatment procedures have led to a generally high level of consistency in commercial antibodies and reagent products from manufacturers.[2–4,39] Satisfactory antibody staining is usually achieved by strictly following the manufacturer's package insert instructions.

TABLE 2. List of Factors That Could be Adjusted During the Antibody Optimization

Parameter	Description
No pretreatment	Some antibodies still perform best without any type of pretreatment
Enzyme digestion	Few antibodies perform best only when enzyme digestion was used without the need for heat induced epitope retrieval
Retrieval buffer	The combination of the type of buffer (ie, citration, ethylenediaminetetraacetic acid, trishydroxymethylaminomethane), and pH level can result in dramatically different signal intensity and signal-to-noise ratio
Heating device	That is, pressure cooker, electronic water bath, microwave, steamer, hot plate
Primary antibody incubation time	This varies depending on the affinity of the antibody to its antigen target, the primary antibody concentration, incubation temperature, and antigen levels in target tissue
Detection system	Polymer detection systems may allow to further dilute the antibody titer, given their generally higher sensitivity than avidin-biotin systems. Tyramine amplification systems are the most sensitive, but also most cumbersome
Chromogen	Prolonging the application of chromogen often lead to more intense signal, but could also compromise the signal-to-noise ratio

Recommendation 5

The test tissue used to optimize the IHC assay should contain the target antigen. Tissue specimens used for antibody optimization should be selected on the basis of the intended use of the IHC clinical assay and the level of target antigen expression in the respective tissue specimen.

Comments

The expression levels of some antigens can vary between neoplasms from different patients, between benign and malignant tissues, and between different types of neoplasms.[53]

Three examples of this issue are provided:

Example 1: the level of CD117 (C-Kit) expressed in gastrointestinal stromal tumors (GISTs) from different patients can range from none to high. Mast cells within GISTs and the adjacent bowel wall strongly express CD117, often at a substantially higher level than the adjacent neoplastic cells. The sensitivity of the CD117 IHC assay should be set such that weak-CD117 expressing GISTs are immunoreactive. If a strongly CD117 expressing GIST or mast cells are used as the test tissue against which the CD117 antibody is optimized, weakly CD117 expressing GISTs may fall below the threshold of immunoreactivity and be interpreted as CD117-negative.

Example 2: some mantle cell lymphomas have low levels of nuclear BCL1 (cyclin D1) antigen, whereas other mantle cell lymphomas and benign tissues such as breast acini have high levels of BCL1. If one of the latter tissues

is used to optimize the BCL1 antibody, mantle cell lymphomas with low-level BCL1 antigen expression can fall below this immunoreactivity cut-point and appear BCL1-negative.

Example 3: CD10 expression in follicular lymphomas and reactive germinal centers is usually substantially lower than in some carcinomas. If CD10 IHC assay is optimized against a follicular lymphoma or tissue abundant in reactive germinal centers, the same assay could be overly intense and possibly uninterpretable when being used as a marker of renal cell carcinoma or to identify bile canaliculi in hepatocellular adenocarcinoma.

The simplest method of establishing multiple IHC assays of a single antibody across a range of clinically different types of tissue after the IHC assay has been optimized in one type of tissue specimen (see Recommendation 7) is to vary the concentration of the antibody. When prediluted antibodies are used, the duration of antibody incubation or intensity of AR can be manipulated to optimize the IHC assay. Regardless of whether concentrated or prediluted antibody is used in the IHC assay, several different IHC assays should be established when one antibody is being used in multiple clinical settings, which cover a broad range of antigen expression levels.

Recommendation 6

The specific antibody clone selected for the IHC assay should be selected on the basis of intended clinical use of the IHC assay and the established record of immunoreactivity in published studies.

Comment

Antibodies bind to a small region of the target antigen. Different antibody clones bind to regions on a target antigen that may be distinct and separate areas, overlap to some extent, or be nearly identical. For many antigens, there are no detectable differences in immunoreactivity between antibody clones when validated against a limited number of specimens. However, for some antigens, the antigenic binding site of the specific antibody clone is of crucial importance. Examples of this issue include: WT1 in desmoplastic small round cell tumors versus ovarian serous carcinoma and mesothelioma,[58–60] the pattern and extent of membranous and cytoplasmic E-cadherin immunoreactivity in ductal versus lobular breast carcinomas,[61–66] and the marked difference in immunoreactivity between the M2-7C10 and Melan-A/A103 clones of MART1 in the PEComa neoplasm group (perivascular epithelioid cell tumors, angiomyolipoma, lymphangiomyomatosis, and clear cell lung neoplasms) and endocrine cell neoplasms.[67–70]

Recommendation 7

New antibody optimization should include serially testing and modifying if necessary, AR, antibody concentration, and chromogen detection system. Serial tissue sections from one tissue block should be used to facilitate the comparison of stain appearances. A minimum of 2 AR buffer solutions, 5 different antibody concentrations, including at least one above, at, and below the manufacturer's recommended concentration, and at least 2 types or incubation times of chromogen detection systems. Each component procedure should be modified accordingly. The combination of component procedures modifications that results in the most consistent and best immunoreactivity is the optimal IHC assay for an individual antibody.

Comments

Laboratories differ greatly in their fixation and tissue processing factors. There is also a broad spectrum of different types and forms of AR and IHC staining procedures. An antigen often has several antibody clones, produced by different manufacturers, and packaged in several different forms. The combination of component procedure parameters that results in the best IHC stain is unique to each individual laboratory. No single IHC assay produces, consistent, high-quality IHC assays across all antigens, every antibody, and in all types of tissues. Recognition of the inherent variability of IHC makes optimization of new antibodies mandatory, regardless of the extent, type, and complexity of the automated IHC platform used by a laboratory. The optimization process often requires several rounds of testing and modifying the component procedures. Occasionally, an antibody cannot be optimized, regardless of the modifications made to the component procedure parameters. Changing antibody clones will frequently remedy this problem. The avidity of most antibodies is influenced by the pH of the AR buffer solution and diluent and have optimal signal intensity within a relatively narrow pH range.[54,71–75]

AR

Testing of a low-pH (example: citrate at pH = 6) buffer solution and high-pH (example: ethylenediaminetetraacetic acid or ethylenediaminetetraacetic acid-trishydroxymethylaminomethane at pH = 8 or 9) buffer solution is recommended.

Antibody

Serial dilutions of the antibody including at least one antibody concentration above and below the manufacturer's recommended concentration. These antibody concentrations should be applied to at least 2 sets of tissue sections, each of which were antigen retrieved in a different buffer solution. The optimal combination of antibody concentration and AR buffer should be selected. If necessary, additional rounds of serial dilutions of more finely graded antibody concentrations should be performed.

Chromogen Detection System

All antibodies (prediluted ready-to-use or concentrated) should be evaluated in at least 2 types of chromogen detection systems. Some antibodies, including some hybridized rabbit monoclonal antibodies produce a

strong antigenic signal using a modified avidin-biotin or 2-step polymer detection system whereas 1-step polymer detection systems result in a thin weak signal (unpublished data, personal experience).

Recommendation 8

Detailed documentation of the antibody optimization parameters and factors, type of tissue used to optimize the antibody, and the tissue block case number should be maintained in the laboratory manual. Records of the set of conditions used, when and how an assay was modified should be maintained as a part of the laboratory manual pertaining to the individual antibody. It is not unusual, over a period of years for a new clone to be introduced, requiring a new set of assay conditions. For an individual case, laboratories should be able to document what antibody clone and assay conditions were in place when the IHC assay stain was performed.

Antibody Validation

Recommendation 9

For IHC assays that are interpreted as a categorical, positive/negative manner, a minimum of 25 separate tissue specimens (samples) tested by an alternative validated method in the same laboratory or by a validated method performed in another laboratory should be evaluated. At least 10 samples should have high levels of the target antigen, 10 samples should have intermediate to low levels of target antigen, and 5 samples should have no IHC evidence of the target antigen.

Additional validation and more restrictive standardized IHC assay parameters are required for therapeutic predictive IHC assays, such as HER2, estrogen receptor, and progesterone receptor.[76–80] Predictive IHC assays are strongly influenced by factors such as length of formalin fixation.[22,81] Genetically engineered, rabbit monoclonal antibodies, such as clone SP1 ER antibody (Lab Vision Co, Fremont, CA) have greater avidity compared with mouse monoclonal antibodies.[82] Validation must be to tissues with known levels of estrogen receptor protein expression or valid clinical/ therapeutic end point to avoid miscalibration.[83–88] Parallel staining comparison with immunoreactivity of a different but similarly, uncalibrated IHC assay is insufficient in this regard. As complexity of interpretation increases, the number and spectrum of specimens required for validation must increase accordingly.

Comments

Each tissue specimen is considered to be a single tissue sample for validation purposes, regardless of the number of tissue blocks from the case. In this context, multiple tissue blocks from a large neoplasm are considered as a single tissue specimen. Three levels of target antigen are selected to confirm that the signal threshold was set appropriately and define the IHC assay parameters. The purpose for the validation procedure is to evaluate the IHC assays performance across a range of clinical samples in terms of relative specificity and sensitivity of the IHC reaction. For this reason, it is important that the samples used for the validation procedure be handled in terms of the preanalytical variables described above, as close as possible to the clinical samples from the laboratory on which the new test might potentially be offered. Tissue microarrays if available are acceptable for the purpose of validating a new IHC assays, and depending on how they have been constructed, have the potential for the survey of a number of different normal tissues and multiple tumor types with the new IHC procedure.

Recommendation 10

A new IHC assay should show a high level of concordance with the validated assays to which it is being compared. Data should be evaluated and discrepancies investigated to determine their origin. A systematic approach of corrective actions should be taken.

Recommendation 11

A log of the validation specimens' samples and the IHC assay results should be kept with the antibody package insert material in the antibody section of the laboratory manual.

NEW LOTS OF ACTIVE IHC ASSAYS

Recommendation 12

All validated IHC assays need to be completely revalidated if significant changes are made to the assay procedure. When new antibody lots of validated active IHC assays are brought into the laboratory, 3 validation test samples should be tested. At least 1 sample should have a high level of the target antigen, 1 sample should have intermediate to low level of target antigen, and 1 sample should have no IHC evidence of the target antigen. Validation of new antibody lots should be sufficient to determine the performance parameters of the IHC assay.

POSTANALYTIC FACTORS

Interpreting IHC Assays

Recommendation 13

IHC assay immunoreactivity should be assessed with a quantitative scoring system.[5,84] All scoring systems should have 2 separate components: the extent or proportion of the target antigen that was immunoreactive and the intensity of the immunoreactivity. Immunoreactivity of an IHC assay should be assessed first. After this assessment, a positive result cut-point threshold should be applied to the result that is clinically appropriate (ie, contextual) and takes into account the expected level of immunoreactivity, specimen size, and amount of target antigen in the specimen.

Comment

There is no universal IHC scoring system. An example of a results classification system with broad clinical utility and used by several members of the committee is to classify 0% to 20% immunoreactivity as "focally reactive," 21% to 80% as "variably reactive," and > 80% immunoreactivity as "uniformly reactive." Another system being used in clinical predictive IHC assays is the IHC score (range, 0 to 400) which is the product of staining intensity (range, 1 to 4) and percentage of immunoreactive target antigens.[89–92] There is also no universal cut-point threshold for a positive result that can be applied across all clinical situations and types of specimens. Substantial variability in interpretation is introduced by the clinical situation, type of specimen, amount of available target tissue in the specimen, and adequacy of specimen fixation and processing.[93]

Interpreting IHC assays in small specimens differs from their interpretation in large specimens.[94] In small specimens, the extent and intensity of immunoreactivity should be assessed in the regional area with the highest proportion of target immunoreactivity. The positive result cut-point should increase (right shift) as the amount of the target antigen available for interpretation decreases. For low-sensitivity antibodies or focally expressed antigens, any level of immunoreactivity can be significant. Numerous factors can complicate the determination of whether an IHC assay result is classified as positive or negative. Separating the assessment and scoring of immunoreactivity assessment from the determination of whether an IHC assay result is positive or negative is recommended to improve reproducibility and accuracy of IHC assay interpretation.

Reporting IHC Assays

Recommendation 14

The following information should be included in the IHC assay report. Each antibody should be reported separately.

1. Antibody clone. Given that the performance of different antibody clones against the same antigen can vary significantly affecting the end result of immunoreactivity, it is critical to report the clone name and type of antibody (polyclonal or monoclonal). For polyclonal antibodies, reference to the vendor's catalog number should be provided.
2. The subcellular localization (nucleus, cytoplasm), which is immunoreactive, pattern of antigen expression (granular, dot, linear, homogeneous), intensity of immunoreactivity within the cellular compartment, and the proportion of cells demonstrating this pattern of immunoreactivity.
3. The scoring system that was applied and the immunoreactivity cut-point threshold of a positive result.
4. Whether there is an internal positive control cell or structure present on the test (patient) slide and descriptive documentation regarding the intensity and proportion of internal control cells or structures that were immunoreactive.
5. Specimen number and block used for IHC slides.
6. Type of fixative and length of fixation.
7. Tissue specimen anatomic location and type of specimen.

Comments

Ideally, all IHC assays would be interpreted using a single, universal standardized scoring system.[75] The different cellular and structural antigenic targets, broad spectrum of clinical situations in which IHC is used, and the lack of consistent, reliable, external and internal control standards, makes this an unrealistic goal.[95,96] Many oncologic-related nuclear regulator antigens are normally present in the nucleus and cytoplasm. It is important that pathologists understand the transport pathways of these proteins and restrict interpretation to the relevant subcellular structures as reported in the literature by that specific antibody clone.[97–99] In the absence of a universal IHC assay scoring system, responsibility rests with the pathologist to provide this information in the report. Documentation also provides a method of verifying the interpreting pathologist evaluated immunoreactivity in the appropriate antigenic target and applied appropriate cut-points thresholds.

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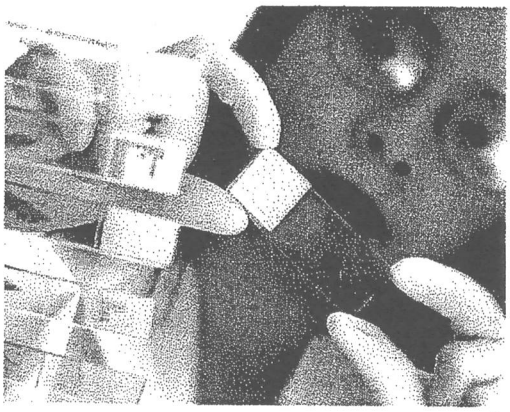
Q&A

Ask the Experts:
On Immunohistochemistry Standardization

The Interviews are based on the Review Article; Recommendations for Improved Standardization of Immunohistochemistry. Applied Immunohistochemistry & Molecular Morphology. 15(2):124-133, June 2007. Goldstein, Neal S. MD; Hewitt, Stephen M. MD, PhD; Taylor, Clive R. MD, DPhil; Yaziji, Hadi MD; Hicks, David G. MD; Members of the Ad-Hoc Committee On Immunohistochemistry Standardization.



Identification of a Slide for analysis

Connection: ***In your first recommendation, you say that non-formalin fixatives are strongly discouraged. However, Formalin's primary ingredient, formaldehyde, has been declared a possible human carcinogen by the Environmental Protection Agency and by the Occupational Health and Safety Administration. Studies have also found that formaldehyde impairs the sense of smell and the respiratory system, irritates the eyes and skin, causes migraine headaches and causes cancer in laboratory animals. Would you still recommend Formaldehyde as the fixative of choice and why?***

Dr. C. Taylor: Yes, formalin has these well known drawbacks, and should be used carefully. That being said – it is the 'best' fixative for regular morphology in that it is forgiving in time of exposure, produces morphologic features that pathologists are trained to interpret, and is cheap.

Dr. H. Yaziji: Until a viable alternative is available, formaldehyde should continue to be the first and only fixative for clinical specimens. The above precautions are real; however, with proper ventilation, utilization and disposal, these risks are minimal.

Dr. S. Hewitt: Yes. Do not think I love formalin. It stinks and burns, but compared to the alternatives it offers the best trade-offs in economics and efficiency. You have to look at the big picture. There is some diagnosis that requires formalin for the cytologic artifacts. One example is the characteristic nuclei of papillary carcinoma of the thyroid. Step back and look at how a lab runs, you need a fixative that is flexible enough to deal with specimens that range from biopsies to organs.

> "Until a viable alternative is available, formaldehyde should continue to be the first and only fixative for clinical specimens."

Times vary in fixation. The closest I have seen was in 70% ethanol and it has limitations. Then you also have to consider cost - both to purchase the fixative and to dispose of the fixative. Is it flammable? How long is it stable on the shelf? If it is, your costs escalate. With formalin, you need good ventilation, but that is it. We have over a century of experience with formalin. It is going to be hard to unseat, especially when you look at all the costs and variables, and appreciate what a low margin business pathology is, and what a small fraction of specimens actually will ever have any kind of molecular analysis (including IHC).

Dr. S. Badve: Even water can cause death if one drinks too much of it. It is important to remember that nearly all the solutions that we use in the lab are toxic in some form or the other. It is imperative to use safe practices at work irrespective of the kind of substances one handles in the lab.

The main question is whether the toxicity is manageable or not. Good ventilation and not working with very strong solutions is the key to dealing with formalin. From the administrative and cost stand-point it is very cheap and easy to dispose off.

Far more importantly than the points above is the fact that we have used formalin for years and years. We know it is not an ideal fixative, however, over the years we have got used to the artifacts it creates and learned to interpret the morphology in a more or less consistent manner. Additionally, all the immunohistochemistry data related to reactivity of antibodies is based on standard formalin fixation. This becomes relevant not only while diagnosing relatively rare lesions but understanding the uncommon reactivity of common antibodies such as keratins in malignant melanoma.

"The more fixatives you add to a laboratory, the more complicated you make it for everyone"

As we go marching ahead into the era of predictive and prognostic markers and associated targeted therapies, it is going to be even more important to have uniform fixation and processing standards. As highlighted by the poster child of targeted therapy, detection of HER2 expression is significantly influenced by fixation. For this reason it is mandated by the FDA that HER2 testing (by IHC or FISH) should be performed on formalin fixed tissues. More recently, the ASCO-CAP (American Society of Clinical Oncology- College of American Pathologists) guidelines have reiterated this view.

Lastly, we know from our experience with various antigen retrieval solutions that the exact constituents of the solutions matter. Not all low pH citrate-based antigen retrieval solutions perform the same. For a given antibody (and lab) one brand can work better than the other. The same might be true for buffered formalin; at the current time, effect(s) of additives to formalin has not been adequately studied.

Connection: ***Is it true that a particular histology feature may be better demonstrated by other fixatives, such as Glyo-Fixx for nuclear features and lymphocyte appearance and Omnifix for cytoplasmic detail (Arch Pathol Lab Med. 2005; 129: 502-506). Would you recommend these fixatives in the future?***

Dr. C. Taylor: NO

Dr. H. Yaziji: No. There is sufficient evidence to discourage the use of glyoxal-based fixatives because of detrimental effects on FISH (and to a lesser degree) IHC testing.

Dr. S. Hewitt: The claims are narrow. If I really wanted to make claims about cytologic features, I would be using B5 for lymph nodes and 70% ethanol for prostate. But that is not a reasonable approach. You need a broad diagnostic fixative. Specialty fixatives are just that. B5 is gone because of the mercury. 70% ethanol is not flammable and actually is easy to integrate, but makes lymph nodes look horrible.

The more fixatives you add to a laboratory, the more complicated you make it for everyone - histotechnicians, pathologist, immunohistochemist.

Dr. S. Badve: It is true that morphology with several fixatives is better than neutral buffered formalin. The hematopathologists have known it for years and were using formalin containing metals such as zinc or mercury to enhance the morphology. However, it was soon realized that these fixatives gave rise to problems when performing IHC analyses and most places have stopped using these fixatives. The simple truth, to put it rather bluntly is, "formalin is the devil you know"

Connection: ***Can you comment of the molecular mechanism of Formalin Fixation***

Dr. C. Taylor: Not really well understood, certainly by me, but also by pathologists in general. The general working concept involves 'cross linking' of protein groups by the aldehyde derivatives, and while I have published in this area (immunomicroscopy - A Diagnostic Tool for Surgical Pathologists, Taylor CR , Cote RJ, Saunders 2006, and in Antigen retrieval techniques, Shi SR Gu J Taylor CR. Eaton Press 2000), I make no pretence to understand the chemistry.

Dr. H. Yaziji: Can you clarify the question a bit more?

Connection: ***How do Formaldehyde molecules act on tissues/cells to make it a good fixative? In other words, what makes this chemical a special fixative in your opinion?***

Dr. H. Yaziji: Formaldehyde is not a very good fixative. It infiltrates the tissue very fast, but it has a very slow fixation rate, and it does not really fit the rapid turnaround time needs of the modern world of pathology.

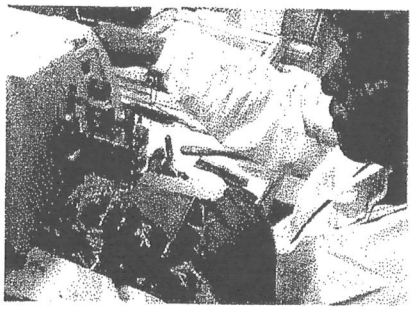
That said, there is no proven better alternative yet, and what makes it a standard fixative is the huge body of literature on predictive markers of thousands of formalin-fixed specimens. That is what gives formalin the advantage over other fixatives, none of which has been documented and/or tested to the extent that formalin has.

Dr. S. Hewitt: Not as well as the papers can. What I can say is that I do not think we actually have the chemistry completely understood, and users underappreciated the impact of different buffers in their formalin, or the impact of old, salted-out formalin.

Dr. S. Badve: The exact mechanism and pathophysiology of fixation is poorly understood, and results on a given protein are unpredictable. Formalin is a solution containing formaldehyde which dissociates at a slow rate. Upon immersion of tissue in formalin, it interacts with formaldehyde resulting in a gradual "fixation". The speed of this process is notoriously slow, partly due to the poor penetration of water soluble formaldehyde through cellular lipids. Hence the common observation, that fatty tissues (such as breast) are difficult to fix and require longer exposure to the fixative.

Connection: ***In your comment regarding recommendation two, you say that tissue processing is not a black box process and that "changes in the processing system have the capacity to alter the chemical makeup of the tissue specimen". Could you elaborate on the changes in the "chemical makeup" (or molecular alterations) of the tissue?***

Dr. C. Taylor: Again, in simple terms, the fact that chemical cross-linking occurs almost certainly affects the folding of the protein molecule, the tertiary structure, if you will. These structural changes in turn affect the recognition and binding ability of an antibody on formalin fixed tissue, because the antibody binds to its antigen through a 3-D charge/structure relationship. The idea is that AR may reverse, in part, these changes.



Sectioning with a microtome

Dr. H. Yaziji: I don't know the exact answer to this question.

Dr. S. Hewitt: "Tissue Processing" is three steps - dehydration, clearing and impregnation, and the latter steps are dependent on the former. If you look carefully, you can define differences in the biomolecules recovered from paraffin embedded tissue based on the process. This is easiest at the RNA level, but we can see it at the protein level. However you can not divorce this from the steps of preparing the tissue and fixing the tissue. They are all related.

Dr. S. Badve: Standard processing protocols entail the use of alcohols after the tissue is fixed in formalin. Alcohol is also a fixative with relatively rapid penetration. So if unfixed tissues are "loaded' onto a processor, they are more likely to undergo significant "alcoholic" fixation which can lead to altered IHC and false positive results.

Connection: ***In recommendation three (Antigen Retrieval) you recommend one AR protocol. Which protocol would be your number one recommendation? How would you standardize this method with so many variations?***

> "So far no one has enforced any rule about fixation; and no one has really tried"

Dr. C. Taylor: In general citrate pH 6.0, 20 minutes, 100 °C; but some antigens do better under different conditions. We like to do a "test battery" of a few protocols to find the best one for each antibody/antigen pair.

Dr. H. Yaziji: The most popular AR that works on the overwhelming majority of antibodies worldwide is heating the tissue in citrate pH 6.

Dr. S. Hewitt: There are better people to ask this one than me.

Dr. S. Badve: There is no one protocol that works for all antibodies. Most labs, (including those that use black box technology i.e. fully automated systems where you load the unstained slides and program the machine to do all the staining without any additional input from humans) use more than one protocol. A useful method when introducing a new antibody is to run the positive and negative control slides with all the AR protocols available in the lab and select the one that works the best for your local conditions (with the least concentration of a given antibody).

Connection: ***Despite the popularity of antigen-retrieval (AR) techniques, the precise molecular mechanism underlying the process remains enigmatic. What is the most understood AR technique?***

Dr. C. Taylor: Don't think any one is most understood.

Dr. H. Yaziji: Like you said, they are mostly speculations without solid evidence. The cross-linking hypothesis is the most popular one, but again without solid proof.

Dr. S. Hewitt: AR is certainly shrouded in mystery. I view it as simple heat time and pressure. Go back to physical chemistry.

Dr. S. Badve: Akin to fixation, the process(es) associated with AR are poorly understood. Although the changes seem to be different for various proteins, the overall process seems to be relatively specific for a given protein.

Connection: ***How would you like to enforce recommendation four through ten? In other words, who will oversee these recommendations and enforce the rules (Food and Drug Administration (FDA), American Society for Clinical Pathology, College of American Pathologists (CAP).***

Dr. C. Taylor: Well so far no one has enforced any rule about fixation; and no one has really tried. CAP and others have recently made a limited set of recommendations, limited in turn to Herceptin; and CAP will try to enforce it starting January 1st. The most likely outcome will be to stop a lot of labs from doing the test; and nothing that is done prospectively will address all the "test data" currently out there.

Dr. H. Yaziji: I hope the pathology societies (CAP, perhaps) will enforce them without interference from the federal government. If we don't self-enforce them, they will be enforced by outside agencies such as the FDA, Clinical Laboratory Improvement Amendments/ Centers for Medicare & Medicaid Services.

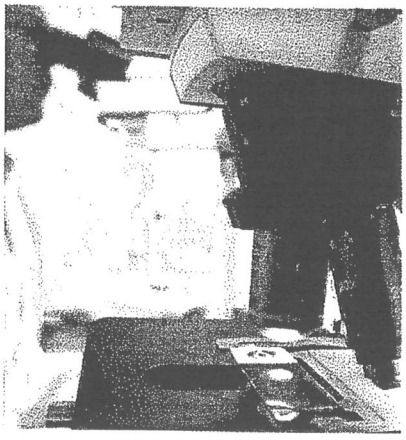
Dr. S. Hewitt: No comment. I work for the government.

Dr. S. Badve: The recommendations are based on good practices and like morality cannot be legislated. This is not for the want of trying. There are several problems that one will encounter if one were to attempt to legislate. Let's take a Keratin antibody for instance, several vendors make these antibodies. Who is going to be the "decider" who proclaims one clone to be better than the rest and mandates its use to the exclusion of all others. This is not possible in a free society. It also assumes that we know and have the means to judge a product as good or bad i.e. we have a GOLD standard. The sad truth is that gold standards are rare in clinical practice. To go back to the classic example of HER2, where response to therapy can be possibly used as a parameter to develop a gold standard, we still do not have a consensus as to which technique (IHC or FISH) is better in predicting likelihood of response.

Connection: ***Recommendation 13: How would you envision the development of a universal IHC scoring system?***

Dr. C. Taylor: Can only see that happening by automation; and then one antigen at a time.

Dr. H. Yaziji: A simple and practical one is semi-quantitative (focally positive, variably positive, uniformly positive). For predictive factors, this is different and should be decided on a marker-by-marker basis. But for non-predictive markers, the above system should suffice.



Observation under a bright field microscope

Dr. S. Hewitt: I do not. I envision objective scoring systems based on function of the assay.

Dr. S. Badve: Universal IHC scoring system is a good theoretical concept; however, it is often not practical. Data from the quality assurance studies conducted by the CAP and by United Kingdom National External Quality Assessment Service (NEQAS) have shown significant variation in the results obtained in different labs on more or less identical material. Depending on the lab the results can vary from negative to weak positive to strong positive even for relatively common antibodies such as estrogen receptor. The reasons for this variation include the pre-analytical variables which generate a need for major differences in IHC protocols.

Connection: ***Recommendation 14: Would a template/procedure/scoring system for reporting IHC standardization assays be beneficial. Do you recommend it?***

Dr. C. Taylor: See above – specifics will have to be 'specific' for each test; some general approaches may have merit as recommendations.

Dr. H. Yaziji: Yes, and yes.

Dr. S. Hewitt: Yes

Dr. S. Badve: Any kind of standard format is better than not having one. It might be easier for predictive and prognostic factors ,but most of the other reagents defining true positive is difficult. When using IHC for classifying a tumor of uncertain histogenesis, the definition of positive varies significantly with the context. Many pathologists will call a tumor spindle cell skin tumor keratin positive even if only a few cells express keratins and particularly, if the tumor lacks positivity for S100 or HMB-45.

> "As more predictive assays come about, there will be greater pressure to standardize tissue handling"

Connection: ***When do you expect these recommendations to become effective?***

Dr. C. Taylor: I couldn't guess; but not soon, except on a test-by-test basis.

Dr. H. Yaziji: When they trickle down to regulatory agencies (such as Clinical and Laboratory Standards Institute (CLSI), FDA, CAP).

Dr. S. Hewitt: Yes, I think this will happen.

Dr. S. Badve: Any kind of standard format is better than not having one. It might be easier for predictive and prognostic factors, but most of the other reagents defining true positive is difficult. When using IHC for classifying a tumor of uncertain histogenesis, the definition of positive varies significantly with the context.

Many pathologists will call a tumor spindle cell skin tumor keratin positive even if only a few cells express keratins and particularly, if the tumor lacks positivity for S100 or HMB-45.

Connection: ***What are the emerging issues that might affect IHC Standardization (Automation, Robotics, Outsourcing)?***

Dr. C. Taylor: You named the three most important. In my view, automation of the "total test" is vital, plus image analysis for scoring. As it gets to be difficult and/ or expensive to meet the elevated standards, more and more tests will be sent to reference labs just as occurred in clinical pathology.

Dr. H. Yaziji: Can you be more specific? Connection: In a few years Robots/ Instruments may replace people to do the routine fixation, staining etc., (like the automobile industry). Also, some of the routine procedures such as mounting may be outsourced to India or China. Who will enforce the rules in these countries?

Dr. H. Yaziji: It's possible that some private equity firm will fund a major quest-like mega lab in India. I am not sure, if this is allowed/ allowable by law. Assuming it's legally feasible to outsource entire patient samples for pathology and laboratory processing, it becomes a logistical nightmare for the CAP (and other regulatory agencies) to ensure that these off-shore laboratories are doing what they are supposed to be doing. In my opinion, the module of outsourcing manufacturing jobs to China and India, if we can draw any valid conclusions from that module, does not carry good news for standardization of tissue processing, IHC and molecular procedures.

Dr. S. Hewitt: Robotics and automation are already here. Outsourcing, in the correct environment offers the capacity to improve immunohistochemistry. Volume can drive quality. Certainly when volume is too low, validation of assays is challenging. I believe "pre-analytic" issues remain the big issue. As more predictive assays come about, there will be greater pressure to standardize tissue handling. This is clearly counter to the push to see more diversity of fixatives and processors. We need objective data on quality. Histology is not an objective measure of quality. If we are going to have personalized medicine, then quality will be measured in the capacity to measure the biomolecules in tissue. This must be objective. IHC is certainly the first platform in clinical labs that enables this.

> "Who will pay for this new level of test performances-because it will cost more."

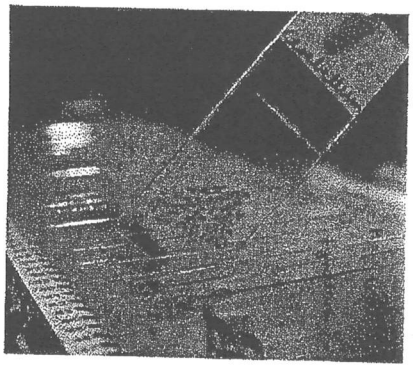
Dr. S. Badve: Automation and robotics is a double-edged sword. Although it may enable/promote standardization of practices, it might at the same time introduce novel variables. These might be in the form of alternative fixatives or use propriety fluids which contain additives; the effects of these may not be obvious for a period of time.

Connection: ***If it is not asking too much - could you tell us how you would address the concerns of those who believe that these rules are difficult to implement?***

Dr. C. Taylor: Can't really answer this; I think that the 'rules' will be introduced piece meal – and will evolve and broaden as labs either adapt or give up and send out the assays. So it will be difficult; but if the payers only pay for properly performed tests, then that will solve the problem quickly!

In my opinion, the biggest and most important unanswered question (because it has not been raised at the highest levels) is who will pay for this new level of test performance? – because it will cost more.

Dr. H. Yaziji: Anatomic pathology is like baseball. All the rules and enforcements have been subjective. It is time for the pathology community to treat anatomic pathology the same way they treat clinical pathology before it's too late.



A stained tissue on a glass slide

Dr. S. Hewitt: I am listening, but I am also listening to the demands of clinicians and patients. I think the biggest challenge is economic. They want more and do not have plans for paying more. Well that is not going to work.

If they want more, and I do believe more information is warranted, then reimbursements have to rise to cover the costs- this is across the board. You do not know what specimens will need molecular testing, and it is not feasible only to pay additional money for those that do.

The model needs to be - Pathology - providing more information for better patient care. In the end it makes better sense. Invest in pathology and personalized medicine, and you will not have to give trials of ineffective, expensive drugs. A penny saved is a pound earned.

Dr. S. Badve: These recommendations have been made by practicing surgical pathologists. They range from the mundane to things we all agree are great goals to have, but will be difficult to implement. However, it is important to remember these recommendations are made by an ad hoc committee with no regulatory powers. They should be seen as a map of where things are and where we need to be in order to provide optimal quality and consistency of lab results; something every lab director strives to achieve.

Connection: ***How would you rate European, Asia-Pacific and Latin American IHC Standards? Are your recommendations followed in these countries? Who sets the standards in these countries?***

Dr. C. Taylor: UK NEQAS, in my view, is ahead of the field in most areas, but not all. They still don't really tackle fixation; and they don't have a good enforcement – police force in place.

Dr. H. Yaziji: The best QC methods in standardizing IHC are across the Atlantic and north of the border. There's ample data to suggest that the CAP proficiency testing doesn't come close to the European and Canadian systems.

Dr. S. Hewitt: Yes and no. Some medicine is international, but there are local variations. Overseas, you do not see the use of vacuum processors as commonly, and the paraffins are at higher melting temperatures commonly. I would anticipate some of them would be adopted.

Dr. S. Badve: The most well known quality assurance program is the UK NEQAS. Similar programs have been set up by many countries with variable results. Traditionally these have been set up by pathologists or pathology societies and the participation has been voluntary with little "regulatory activity". With the advent of targeted therapy, other bodies such as ASCO are also getting involved in ensuring quality assurance and quality control. One of the major issues that frequently arise is the availability of adequate control material for use in proficiency testing.

> "I feel that I have a responsibility to share the accumulative knowledge that I've learned over the years..."

Connection: ***As a leader in the industry, how have you kept up with the changes?***

Dr. C. Taylor: I talk to people, read the literature, attend selected meetings that appear key to me, and talk to some more people. No one individual, or one group or organization, can make significant change here; this requires a co-ordinated and global response. It is starting; but will take time; it has taken more than 20 years to date, and may take 20 more. Hope I can still read.

Dr. H. Yaziji: Yes, it is always challenging for anyone to balance between their family duties and their academic career. I can't claim that I am very successful in keeping this balance under control. On the professional side, I feel that I have a responsibility to share the accumulative knowledge that I've learned over the years with the community that needs it most.

What's worse, is that the more knowledge we acquire, the heavier the burden gets. I, like every academician, feel that it is my duty to contribute to the community what I think is useful. My consulting company is doing just that, and I hope that our work speaks for itself. Certainly, we are having fun doing that and I am truly fortunate that I'm part of a very interesting activity revolving around cutting-edge issues related to ancillary testing in pathology.

Dr. S. Hewitt: It is a chronic challenge. You will actually see me in the booths a meetings kicking the tires on instruments, and trying reagents for the fun of it, when I can afford to. We borrow and collaborate with other groups. My interest is very bio-specimen driven and less disease specific, so we tend to do lots of collaborations.

Dr. S. Badve: There are major developments going on in the field and it is impossible to keep up with all aspects of scientific and technological developments. I have learnt a lot during the discussion we had at this committee meeting. As in anything else, getting involved is an important part of the solution.

Connection: ***Thank you for taking the time to give us your opinions.***

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