Chapter 1
Immunohistochemistry Quality Management and Regulation

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Abstract Immunohistochemistry testing is highly complex with multiple steps. Assuring the optimum performance of your immunohistochemistry laboratory requires attention to numerous quality monitors. For testing performed on patient specimens, there are also additional regulatory requirements. This chapter answers questions about best practices in quality management in preanalytic, analytic, and postanalytic phases of the total immunohistochemistry test providing examples of possible quality improvement opportunities. It also provides information related to CLIA and FDA regulatory oversight medical devices, in vitro diagnostics (IVD), and analyte-specific reagents (ASR). With regard to immunohistochemistry laboratory accreditation, the final portion of this chapter draws attention to current best practice guidelines of the College of American Pathologists (CAP) relating to immunohistochemistry to prepare for inspection.

Keywords Quality • Regulation • CLIA • FDA • ASR • IVD • Controls • Validation • Inspection

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IHC Quality Management

1.1 What Should Be the Scope and Significance of a Quality Management Program for Immunohistochemistry (IHC)?

Although immunohistochemistry (IHC) is a staining procedure, the factors that affect the quality of the results include events spanning from the identification of the specimen to the presentation in the report of the significance of the result to the submitting physician. So a program to manage the quality of IHC should address issues spanning the pre-analytic, analytic and post-analytic spectrum of the total testing process. Best practices should be implemented and processes monitored to detect and correct deficiencies in order to produce the best results. The need for quality results in IHC has only increased as the use of these tests has evolved from being markers of tumor differentiation to now include being predictive markers guiding the use of specific therapies. IHC stains are now, more than ever, an integral part of the practice of anatomic pathology. However, current and projected future healthcare economics make obvious the need for cost containment through comprehensive analysis and continuous quality improvements of workflow processes and appropriate utilization of IHC resources.

References: [1–11].

1.2 How Does Specimen Identification Affect IHC Quality?

The only way to provide a correct result for the correct patient it to ensure correct labeling of the specimen beginning from the initial acquisition of the specimen in the clinician's procedure room or operating room. To avoid confusion if specimen requisitions are separated from the specimen containers, both should be legibly labeled with at least two patient identifiers and the specimen type and location. This requirement for specimen identification should be monitored and enforced with submitting locations in order to emphasize the importance. Non-compliant specimen labels should be investigated to satisfactory resolution of identity with the submitting site identity or else rejected. The Joint Commission on Accreditation of Healthcare Organizations (JCAHO) has made the use of two patient identifiers a National Patient Safety Goal applicable to laboratories. Modification of the requisition form may be necessary to help sites comply with specimen labeling requirements. Instances of problems with specimen labeling should be tracked and quantified in order to direct customer education resources to the most needed sites. Bar-coding can be a major factor in reducing misidentification errors in anatomic pathology.

References: [11–17].

1.3 How Does Specimen Handling Relate to IHC Quality?

The topic of tissue handing and its affect on IHC testing has become common since the release in early 2007 of the

American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines for the Her-2 testing. The specifics of those guidelines will be discussed elsewhere in the book. But the inclusion of specific specimen handling recommendations in that report reinforces their importance.

The key issue in pre-analytic specimen handling is to quickly get the tissue into standardized fixative to reduce the ischemic time until fixation and prepare the tissue for your validated antigen retrieval methods.

It is obvious that chemical breakdown resulting from ischemia would interfere in the detection of biomarkers in specimens. Although very stable markers such as DNA and intermediate filaments are able to be detected in necrotic tissue, other markers are far less resilient. Ischemic degradation is most noted with fragile mRNA molecules intended in vivo to be only fleetingly present to deliver their transcriptional messages. Breakdown of these molecules can be seen in a matter of minutes. CAP recommends limiting ischemic time for breast tissue specimens to be used for receptor studies to be less than or equal to an hour.

Ischemic degradation of tissue is halted by the process of fixation by chemically stabilizing molecular structures which creates linkages in the proteins. This has the effect of paralyzing tissue enzymes in addition to other proteins which stops autolysis. Different fixative solutions have different times of tissue penetration and rates of fixation. Therefore, larger specimens should be refrigerated if dissection is to be delayed. And when dissected, tissue sections should be thin enough so as not to be compressed by the cassette lid, which restricts fixative penetration. If breast tissue from a large resection is to be submitted for critical receptor studies, consideration should be given to either incising the tumor to expose the surface to fixative or submitting a single tissue section from the tumor prior to completing the full dissection.

Some biomarkers are affected differently by the use of different fixatives. An example of this is a loss of expression of S100 by IHC in tissue fixed in alcohol compared to the same tissue fixed in formalin. The effects of differences in fixation are not known for most biomarkers. And tissue fixation is probably the most out-of-control variable affecting the quality of IHC staining. So the best practice is to attempt to standardize the type and time of fixation used for tissues in your laboratory in order to optimize your antigen retrieval protocols to these fixation conditions. Methods recommended to standardize fixation in the pre-analytic phase of testing include using only 10 % neutral buffered formalin (NBF). Formalin is not universally accepted to be the best fixative for all tissue types, but is the most commonly used fixative and provides for adequate histologic preparations for most antigens. Requiring formalin in your specimen submission requirements can help achieve this goal. Of course alternative fixatives may be considered satisfactory if the laboratory has performed validations of their IHC testing protocols using these alternative fixatives.

The other side of quickly placing tissues into fixative is controlling how long the tissues spend in the fixative solution. Tissues will be subjected to standardized antigen retrieval protocols designed to breakdown the bonds created by fixation. If tissue is inadequately fixed to withstand this retrieval process, target proteins may instead by destroyed, resulting in false-negative IHC results. This is known to occur with estrogen receptor protein testing performed on tissues fixed in formalin for less than 6 h. Tissues that are overfixed may also be falsely negative due to inadequacy of the standardized antigen retrieval protocol to reverse the effects of prolonged formalin fixation. In our experience, this is less commonly an issue with modern antigen retrieval methods. Each laboratory should have a procedure to control the minimum and maximum time tissues spend in fixative prior to processing and embedding. It is recommended that ischemic time and fixation type and time be recorded for tissues submitted for breast cancer receptor studies. Many laboratories have modified their specimen requisitions by providing an area of the form specifically for entering this data.

References: [18–39].

1.4 How Can I Assure the Qualifications of IHC Testing Personnel?

Histotechnologists (HTs) have the certification required to perform IHC testing, though the level of experience of histotechnologists with IHC varies greatly. The American Society of Clinical Pathology (ASCP) offers an additional certificate program for histotechnologists verifying advanced knowledge of the theory behind IHC testing as well as practical experience with optimization and performance of IHC. What is most critical is that staff have a familiarity with appropriate and inappropriate control reactions (non-specific stromal staining, endogenous peroxide and biotin, staining artifacts, sub-cellular compartment of signal, tissue pigments) and are able to recognize tissue artifacts before releasing slides to the pathologist. Competency testing of testing personnel should be performed and documented annually. Delays in recognition of poor quality staining lead to delays in rerunning stains to produce adequate results. Such delays only serve to delay the final reports to the clinicians. The number of poor stains released should be monitored to direct re-education of staff. Providing images and descriptions of expected positive and negative staining patterns for each in-house stain can benefit histotechnologists as well as pathologists.

References: [4, 40–43].

1.5 What Role Can Research Literature Play in Optimizing an IHC Assay?

The first step to producing a clinically useful and valid IHC assays is by choosing clinically relevant and technically superior antibodies and reagents in your testing system. Our best advice is to review the literature to determine which antibody clones have associated clinical significance with reproducible protocols. Often requests for bringing on new antibodies are based on articles in the literature for a specific clinical application. In these cases it would be advisable to acquire a copy of the article from the requesting pathologist or clinician to determine the clone and assay parameter used in the study in order to reproduce them as closely as possible in your laboratory. Even if the article does not provide sufficient information to reproduce the testing results, contacting the corresponding author is often fruitful. Otherwise, the article should at least indicate which tissue should produce positive and negative results so that these can be used to optimize the assay in your laboratory.

References: [2, 4, 44, 45].

1.6 How Should RUO, ASR and IVD Designations Be Considered in Selecting Antibodies for Optimizing an IHC Assay?

Another consideration for choosing a clone is to determine which reagent class an antibody falls into. Antibodies developed in laboratories and not submitted to the FDA for approval are designated as Research Use Only (RUO). As vendors pay for and accumulate research so that they are able to demonstrate increasingly reliable performance characteristics for their antibodies to the FDA, they received designations as either Analyte Specific Reagents (ASR) or, for the most fully characterized antibodies, there is a designation as an In Vitro Diagnostics (IVD). As vendors collect this research and obtain these higher class designations, they are able to supply more information. Datasheets for IVDs can contain more information regarding the expected performance of antibodies in their accompanying datasheets, often listing normal and abnormal tissue reactivities indicating tissue types for optimization and control tissues. CAP-accredited laboratories have established rules for using RUO reagents. According to CAP guidelines, RUOs may only be used when no other class of antibody is available. RUOs purchased from commercial sources may be used in laboratory-developed tests only if the laboratory has made a reasonable effort to search for IVD- or ASR-class reagents and the results of that failed search are documented by the laboratory director. If a CAP accredited laboratory performs patient testing using Class I ASRs obtained or purchased from an outside vendor, federal regulations require that a disclaimer accompany the test result on the patient report stating, "This test was developed and its performance characteristics determined by (laboratory name). It has not been cleared or approved by the U.S. Food and Drug Administration." CAP recommends adding and additional statement, "The FDA has determined that such clearance or approval is not necessary. This test is used for clinical purposes. It should not be regarded as investigational or for research. This laboratory is certified under the Clinical Laboratory Improvement Amendments (CLIA) as qualified to perform high complexity clinical laboratory testing." Attention to the class designation of antibodies is a Best Practice and CAP guideline.

References: [8, 46, 47].

1.7 How Should I Choose Tissues for Performing an Optimization of an IHC Assay?

Determining which choices to make for each of the steps of an IHC assay to achieve optimum performance is known as optimization. This process can be as simple as reproducing the vendor's recommended protocols on with your equipment on your tissues. Unfortunately, it is the nature of reacting antibodies with fixed tissues that the optimization process is too often a long and confounding experience.

One of the most important keys to successful optimization is choosing the correct tissue. The first point to make is to choose tissues from your own paraffin archive of surgical specimens that were handled as typical specimens on which you would want to run the IHC test for diagnosis. Choosing tissues from autopsy cases can be a mistake if the tissues were allowed to autolyze before fixation or were taken from tissues fixed for a much longer period of time. Autopsy tissues handled so differently from typical patient specimens are unlikely to react similarly or to serve as a good basis for test optimization. Similarly, tissues from other laboratories should not be used for optimization due to potential handling differences.

For markers intended to differentiate between two or more tumor types based on qualitatively positive or negative expression, the tissues chosen for optimization should reflect the positive and negative tissues types in that differential diagnosis. For assays designed to produce quantitative results or used to determine a certain threshold level of positive expression (such as Her-2), tissue used for optimization should be chosen to reflect the range of results on both sides of the diagnostic threshold for that marker.

References: [2, 6, 8, 44, 48].

1.8 What Are the Steps to Vary in Optimizing an IHC Assay for a Chosen Antibody?

Typically, researchers involved in optimizing newly developed primary antibodies or complex multi-antigen detection protocols will have years of experience with testing protocol variations with their open systems and will not be the people asking this question. In most instances, people new to optimizing IHC assays will be using well-characterized antibodies with dilutions recommended by the vendor on automated systems that have predefined detection protocols. In that setting, the choices to be considered in beginning an optimization process have been greatly simplified. For cases where the primary antibody has a recommended dilution from the vendor, attempting that dilution and dilutions at double and half that concentration are good starting points for testing. If the vendor has supplied a prediluted antibody, then the question of primary antibody diluted is moot, so choose a short, brief and long antibody incubation time instead. In either case, attempt these antibody dilutions or incubation times on positive and negative control tissue sections (small sausage blocks are excellent for this purpose) with each of three different retrieval protocols: 1) HIER with pH 6 citrate buffer at 100 °C for 20 min, 2) HIER with pH 8 EDTA buffer at 100 °C for 20 min, 3) and a short 4-min protease digestion. Evaluate the results of these test protocols to determine the best combination of strong specific staining and minimize non-specific background staining. The results of this initial set of tests should provide you with an indication of which direction to take your next optimization experiment. Additional blocking or amplification steps may be necessary to complete your optimization. For additional information related to stain optimization and troubleshooting, refer to Chap. 2.

References: [1–8, 44, 45, 48–54].

1.9 What Are the Steps to Validate an IHC Assay for a Chosen Antibody?

An optimization is a preliminary step to antibody validation during which the optimized protocol is tested to determine sensitivity and specificity of the IHC assay. To achieve this, numerous positive and negative control tissues representing typical specimen handling for your laboratory are obtained from your paraffin archive of cases. These positive and negative cases should reflect the types of tissues for which the test was developed in order to test how the test will perform in the clinical setting. The NCCLS (CLSI) guideline requires IHC testing to undergo a validation, but most of the details of this validation are left to the discretion of the qualified laboratory director [6]. In its laboratory accreditation program guidelines, CAP has cited a commonly referenced article by Hsi [46] regarding the performance of IHC validation. This article suggests testing a minimum of 10 positive and 10 negative cases for well-established antibodies and at least 20 positive and 10 negative cases to determine the sensitivity and specificity of less well-characterized antibodies. An exception is made for very rare antigens such as ALK for which it may be more reasonable to collaborate with other institutions to aggregate enough cases for validation. Or alternatively, perform a prospective validation of you assay's performance in parallel to results obtained from an outside laboratory with an established, validated assay. In this way the test can be introduced clinically based on the outside validation while an internal validation is accumulated.

Newly announced guidelines regarding the validation of breast cancer receptor studies have far more specific requirement and will be addressed in their own chapter.

References: [1–8, 44, 45, 48, 55].

1.10 What Are the Best Control Tissues for IHC Assays?

The best positive and negative control reactions are those present within the patient tissue sample. The best example of this is the presence of weak estrogen receptor (ER) protein expression in the normal breast ducts. If a section is chosen for ER testing to include normal benign duct structures along with tumor, then positive staining of the internal normal ducts is excellent confirmation of a result of negative ER expression within the tumor on the same slide. Similarly, in a CD20 assay that is positive in B cells, the lack of staining of associated T cells is good evidence of the specificity of the positive CD20 reaction in the B cells. Of course, adequate control tissues are not always present on a slide to be tested. Fortunately, as a part of the process of a well-performed validation of an IHC assay's performance, positive and negative control tissues are identified and validated which can be used as controls in the clinical assays. If these are in sufficient supply, then these tissues are the ideal control tissue for the clinical assay. When ideal control tissues are scarce, often normal tissues that are in plentiful supply (e.g., tonsils, endomyometrium, appendix) are substituted as control tissues. A drawback of this choice is that normal tissue often expresses characteristic proteins more strongly than tumor tissues, especially the very poorly differentiated tumors on which IHC assays are often ordered. There is a risk in using these strongly expressing tissues as positive control. In the event that the assay drops significantly in sensitivity, there may still be positive control staining while the weakly

expressing tumor tissue in the patient sample becomes falsely negative. For this reason, control tissues are best when they express at levels at the threshold level of detection for the patient tissues being tested.

Negative controls studies lacking the primary antibody should be performed on sections cut from the patient block in parallel with the assay on the patient tissue to control for non-specific staining. A negative control is required for each detection protocol used in the panel of assay performed on the patient tissue. If multiple types of antigen retrieval protocols are utilized, it is acceptable practice to perform the negative control assay using the retrieval protocol considered to be the most aggressive. Which is the most aggressive is not always clear, but as a general rule higher pH EDTA is considered more aggressive than pH 6 citrate HIER, and the addition of protease is even more aggressive.

References: [1–8, 56–60].

1.11 What Are the Parts of Daily Quality Control in the IHC Test?

The key to quality control in the performance of IHC staining is process standardization, which requires clear standard operating procedures and could benefit from automation (see Chaps. 3–5). Many additional techniques can aid in achieving quality control of the processes. Computer software and hardware utilizing barcode tracking of blocks, slides and reagents can be leveraged to save time and reduce misidentification errors. Some barcoding systems can even offer real-time detection and correction of delays and bottlenecks in workflow due to staffing or equipment failure.

Attention needs to be paid to daily equipment calibration and maintenance. Reagent conditions also require attention with regard to storage and testing temperatures and expiration dates. Lot-to-lot comparisons of new reagents are required to assure equivalent performance to prior reagent lots.

Batch positive controls require review before release to pathologist and must be made available to pathologists if needed. Other positive and negative controls performed along with patient cases should also be reviewed to detect assay failures prior to releasing to pathologists. Review of stain quality before releasing to pathologist detects and corrects staining errors sooner, avoiding delays in reordering and patient results. For this purpose it is also essential to have established rejection criteria for slide acceptability for interpretation (e.g., control failure, mislabeling, background staining, cytoplasmic staining for a nuclear stain or vice versa, extensive edge artifact, lack of tissue adherence to slide, lack of coverslip or insufficient mounting media).

There also needs to be a mechanism to permit feedback from the pathologist to the histotechnologists regarding the status of staining quality as another check on assay performance.

References: [1–8, 12].

1.12 What Are the Staining Artifacts and Failed Control Reactions to be Aware of When Interpreting IHC Assay Results?

Quality control of the interpretation of an IHC slide should begin with the internal and external control reactions. Positive studies should always be confirmed by appropriate negative control reactions and vice versa. Even with appropriate external control reactions, the pathologist should be aware of staining pitfalls related to patient tissue conditions such as false-positive results related to edge artifact, crush artifact, necrosis, endogenous pigments, endogenous biotin or peroxidase, detection of immunoglobulins in plasma cells. False-negative results may occur as a result of poor tissue preservation or non-standard fixation. Uneven staining of patient tissue with appropriate controls should suggest poor tissue processing, and a different block from the case should be used if available.

Mistakes can also be avoided if the pathologist is aware of the expected localization of the staining response and does not accept a positive cytoplasmic reaction as positive for a stain expected to be localized to the nucleus.

Failure of required control reactions should trigger a repeat of the assay, possibly on a different tissue block from the same case. The incidence of repeated stains should be monitored for evidence of a poorly performing assay. Repeated failure of the control study should trigger a thorough investigation of the parameters of the staining protocols and substitution of fresh reagents. Re-calibration and maintenance of the equipment may be required to resolve the issue. When all these steps fail to correct the problem, a re-optimization and re-validation of the assay may be necessary.

References: [1–8, 36, 44, 45, 48, 53, 56–61].

1.13 What Are Some Examples of Quality Assurance Monitors for Analytic Phase of IHC Testing?

1) Monitor turnaround time for stain orders
2) Monitor trends in positive, equivocal and negative results of predictive markers
3) Participation in external proficiency testing and laboratory accreditation inspections

References: [1–8, 38, 39, 62–65].

1.14 What Can Be Monitored in the Post-Analytic Phase of IHC Testing?

Post-signout review of reports can be used to monitor the completeness of required documentation of stain and control results in the report and the accuracy of associated billing for IHC assays. The results of billed IHC testing are required to be documented for each antibody. Occurrences of duplicated billing when the same antibody is run multiple times on the same specimen part can be detected and credited. Identification of these types of mistakes can direct educational efforts and redesign of billing automatically triggered by laboratory information system processes.

References: [4, 5].

1.15 What Are Some Examples of Quality Improvement Opportunities in IHC Testing?

1) Identify root cause of infrequent, though critical zero-tolerance errors (lost or overly faced blocks, mislabeled slides, and tissue contaminants) for interventions
2) Identify common issues causing inefficiencies in the IHC workflow (e.g., coordinate adequate staffing with timing of courier and processor runs, evaluate capacity of manual and automated staining processes for high slide volumes times, monitor IHC repeat orders as rework)
3) Update current equipment and antibody library to meet current clinical testing needs. This can be accomplished by monitoring the literature for newly available antibodies and equipment or new uses for existing antibodies, and by following the migration of your existing antibodies from ASR to IVD status and polyclonal to monoclonal forms. With this information, proceed to validate assays for the use of the most clinically relevant antibody clones
4) Monitor intradepartmental peer ordering patterns to target education for under or over utilization of testing

Reference [4].

IHC Laboratory Regulations

1.16 What Is the Law Regulating IHC Laboratory Testing?

Congress passed the Clinical Laboratory Improvement Amendments (CLIA) in 1988 establishing quality standards for all laboratory testing to ensure the accuracy, reliability and timeliness of patient test results regardless of where the test was performed. CLIA ’88 establishes minimum performance standards for all clinical laboratories with regard to quality standards for proficiency testing (PT), patient test management, quality control, personnel qualifications and quality assurance for laboratories performing moderate and/or high complexity tests. Under CLIA, a laboratory is any facility that does laboratory testing on specimens derived from humans to give information for the diagnosis, prevention, treatment of disease, or impairment of, or assessment of health. In total, CLIA covers approximately 200,000 laboratory entities. CLIA also regulates the manufacturers of commercially available the reagents and instrumentation used for performing IHC and regards these as medical devices.

References: [66–72].

1.17 What Is the Concept of Complexity with Regard to Laboratory Testing Regulation?

Prior to CLIA ’88, regulations regarding laboratory practices varied depending on the type of site (independent, hospital, or physician’s office laboratory) with physician office laboratories loosely controlled. Under CLIA ’88, laboratories in the United States are regulated based on the test complexity rather than by where the test is done. Laboratory tests categorized under CLIA as high complexity may only be performed in laboratories CLIA certified to perform high complexity testing.

References: [67–71].

1.18 What Are the Agencies and Organizations Responsible for Implementing CLIA Regulations for Clinical Laboratories?

The Department of Health and Human Services (DHHS) is responsible for overseeing CLIA rules for all clinical laboratory testing (except research) performed on humans in the United States. The Centers for Medicare & Medicaid Services (CMS), under the DHHS, assumes primary responsibility for financial management operations of the CLIA program. A laboratory must be CLIA certified in order to perform clinical laboratory testing and to receive Medicare payments for testing. The implementation of the CLIA Program regulations has fallen to the CMS Division of Laboratory Services, within the Survey and Certification Group, under the Center for Medicaid and State Operations (CMSO).

The Commission on Office Accreditation (COLA), The Joint Commission on Healthcare Organizations (JCAHO), and the College of American Pathologists (CAP) are non-governmental, professional organizations which have received deemed status from CMS to inspect and certify that laboratories meet the CLIA standards.

CAP's Laboratory Accreditation Program (LAP) is widely recognized as the "gold standard" and has served as a model for various federal, state, and private laboratory accreditation programs throughout the world. CAP accreditation is accepted for both CLIA and JCAHO certification. The CAP inspection program is internationally recognized and the only one of its kind that utilizes teams of practicing laboratory professionals as inspectors. Designed to go well beyond regulatory compliance, the program helps laboratories achieve the highest standards. There are more than 6,000 CAP-accredited laboratories nationwide.

Another non-governmental organization related to laboratory standards, originally known as National Committee for Clinical Laboratory (NCCLS), changed its name to the Clinical and Laboratory Standards Institute (CLSI) in January 2005. CLSI develops and publishes standards and guidelines through a consensus process that involves representatives from government, industry, and the patient-testing professions. CLSI has no regulatory authority of its own, so its standards and guidelines are regarding the performance of IHC are only mandatory when they are referenced by other regulatory organizations such as CAP.

References: [38, 67–75].

1.19 What Are the Agencies and Organizations Responsible for Implementing CLIA Regulations for Manufacturers of IHC Reagents and Instrumentation?

In addition to regulating clinical laboratories, CLIA regulates the manufacturers of commercially available the reagents and instrumentation used for performing IHC and regards these as medical devices generically called In Vitro Diagnostics (IVD). Under CLIA '88, the Food and Drug Adminstration (FDA) Office of In Vitro Diagnostic Device Evaluation and Safety (OIVD) administers the CLIA test complexity program for medical devices. Within the OIVD are the Division of Immunology and Hematology Devices (DIHD) specifically responsible for Tumor marker (cancer detection) tests such which is the most common use for IHC and ISH and the Division of Microbiology Devices (DMD) responsible for any IHC or ISH tests for the detection of microorganisms (bacteria, fungi, mycobacteria, viruses).

References: [67–71].

1.20 How Does CLIA Control the Use of IHC Testing Through Determination of Laboratory and Test Complexity?

The Food and Drug Adminstration (FDA) Office of In Vitro Diagnostic Device Evaluation and Safety (OIVD) of categorizes commercially marketed in vitro diagnostic (IVD) tests by level of complexity based on their potential for risk to public health as 1) waived, 2) moderate or 3) high complexity. IHC testing is considered high complexity testing. Therefore a laboratory must be accredited under CLIA to perform the level of complexity of the testing done in their facility.

References: [67–72].

1.21 How Does CLIA Control the Marketing and Use of IHC Testing Through Test Class?

And like other medical devices, IVDs are subject to premarket and postmarket controls to be determined by the FDA. Before a manufacturer can make an IHC testing reagent, test or instrument commercially available, it must determine the level of premarket documentation of performance characteristics and safety that will be required by the FDA. FDA classifies IVD products into Class I, II, or III according to the level of regulatory control that is necessary to assure safety and effectiveness. The classification of an IVD (or other medical device) determines the appropriate premarket process.

Class I – Class I devices are subject to the least regulatory control. They present minimal potential for harm to the user and are often simpler in design than Class II or Class III devices. Class I devices are subject to "General Controls" as are Class II and Class III devices. General controls include provisions that relate to adulteration; misbranding; device registration and listing; premarket notification; banned devices; notification, including repair, replacement, or refund; records and reports; restricted devices; and good manufacturing practices. The general use of IHC antibodies involving indetermination of tumor differentiation is

regarded as Class I or low risk and is almost always exempt from premarket notification and approval requirements of Class II and Class III IHC antibodies.

Class II – Class II devices are those for which general controls alone are insufficient to assure safety and effectiveness, and existing methods are available to provide such assurances. In addition to complying with general controls, Class II devices are also subject to special controls also known as premarket notification or 510(k). Special controls placed on Class II devices may include special labeling requirements, mandatory performance standards and postmarket surveillance. IHC antibodies for estrogen and progesterone receptor proteins and Her-2 oncoprotein are used in testing to predict the use of hormone based and trastuzumab therapy, and are therefore considered of higher risk than general differentiation markers and fall into Class II requiring 510(k) premarket notification clearance. Automated microscopes for image analysis of IHC are also considered Class II by the FDA. Some vendors seek Class III premarket approval for when it is not required to differentiate their product in the market.

Class III – Class III is the most stringent regulatory category for devices. Class III devices are those for which insufficient information exists to assure safety and effectiveness solely through general or special controls. Class III devices are usually those that support or sustain human life, are of substantial importance in preventing impairment of human health, or which present a potential, unreasonable risk of illness or injury. Premarket approval is the required process of scientific review to ensure the safety and effectiveness of Class III devices. Not all Class III devices require an approved premarket approval application to be marketed. Class III devices which are equivalent to devices legally marketed before May 28, 1976 may be marketed through the premarket notification [510(k)] process until FDA has published a requirement for manufacturers of that generic type of device to submit premarket approval data. A 510(k) requires demonstration of substantial equivalence to another legally U.S. marketed device. A claim of substantial equivalence does not mean the new and predicate devices must be identical. Substantial equivalence means that the new device is at least as safe and effective as the predicate. A device is substantially equivalent if, in comparison to a predicate it has the same intended use as the predicate and has the same technological characteristics as the predicate; or has the same intended use as the predicate and has different technological characteristics and the information submitted to FDA but does not raise new questions of safety and effectiveness and demonstrates that the device is at least as safe and effective as the legally marketed device. In IHC, testing for c-kit and epithelial growth factor receptor (EGFR) used to predict targeted therapies are considered Class III by the FDA and require the premarket approval process.

References: [67–72].

1.22 What Is the FDA's ASR Rule?

ASR stands for Analyte Specific Reagents (ASRs) and is designation for a special subset of IVD reagents created by the FDA to have fewer premarket requirements along with fewer premarket claims of testing performance. The ASR rule recognizes the difference between a general purpose reagent, such as buffers that lack specificity for an analyte, and antibodies or nucleic acid probes that by design have binding specificity for an analyte. It also recognizes the need for a difference between an In Vitro Diagnostic test (IVD) validated and marketed by a vendor and subject to premarket notification requirements (510(k)), and an antibody or probe sold to a CLIA-accredited laboratory used to develop an "in-house" assay to be validated by the laboratory itself exempting the vendor from premarket notification requirements. The FDA created the ASR category as the least burdensome regulatory approach to foster cooperation between vendors and laboratories in developed tests. By accepting the ASR designation, vendors can make antibodies and probes available to laboratories sooner than if they were required to perform the premarket notification process for each antibody as an IVD. ASR rule allows a description of the specific binding of an antibody or probe as long as there is no claim made for the clinical use which would change the antibody from the component of a test into a test itself. As such the FDA requires that clinical testing performed using ASRs provide documentation that the test has not been evaluated by the FDA and the laboratory is certified to perform high complexity testing and is responsible for and has validated the test that uses the ASR. An acceptable ASR disclaimer to satisfy the FDA would be, "This test was developed and its performance characteristics determined by (laboratory name). It has not been cleared or approved by the U.S. Food and Drug Administration."

References: [47, 67–72].

1.23 What Are the Limitations Placed on the Information That a Vendor Can Provide a Laboratory for an ASR Reagent?

Since ASRs are considered specific individual "building blocks" of laboratory developed tests (LDT), a vendor is limited in the information that can be provided to a laboratory.

ASR labeling may indicate the affinity of the reagent to a molecular target, such as "anti-estrogen receptor antibody" or "CFTR nucleic acid probe" because it only describes the ligand to which the ASR is specific but does not claim to

produce a particular clinical or analytical result. ASR manufacturers also should not promote, sell, or otherwise distribute other reagents, software or instrumentation that could imply that such as packaging is needed to achieve a function of an ASR. Vendors should also not assist with the development or validation of an LDT using its specific ASR. Under the CLIA regulations, the laboratory must conduct validation and verification of test performance specifications. 42 CFR 493.1213. This validation by the laboratory is the minimum requirement under CLIA for the laboratory to generate clinical results for tests of high complexity. For ASRs the sole responsibility for how to use the ASR in testing lays with the performing laboratory.

References: [47, 67–71].

1.24 How Is the ASR Rule Related to In Vitro Diagnostic Products Labeled for Research Use only (RUO) or Investigational Use Only (IUO)?

Products labeled for research use only (RUO) or investigational use only (IUO) are IVDs in different stages of development. The FDA considers RUO products to be products that are in the laboratory research phase of development, that is, either basic research or the initial search for potential clinical utility, and not represented as an effective in vitro diagnostic product. These products must be labeled "For Research Use Only. Not for use in diagnostic procedures." as required under 21 CFR 809.10 (c)(2)(i).

FDA considers IUO products to be products that are in the clinical investigation phase of development. They may be exempt from the investigational device (IDE) requirements of 21 CFR Part 812 (21 CFR 812.2(c)), or may be regulated under 21 CFR Part 812 as either a non-significant risk device or a significant risk device. Diagnostic devices exempt from IDE requirements cannot be used for human clinical diagnosis unless the diagnosis is being confirmed by another, medically-established diagnostic product or procedure (21 CFR 812.2(c)(3)(iv)). This is a validation of the performance of the test using the RUO component performed by the laboratory CLIA certified to perform high complexity testing. During this phase, the safety and effectiveness of the product are being studied; i.e., the clinical performance characteristics and expected values are being determined in the intended patient population(s). These products must be labeled, "For Investigational Use Only. The performance characteristics of this product have not been established." 21 CFR 809.10(c)(2)(ii).

References: [47, 67–71].

1.25 What Is the Difference in FDA Requirements for Manufacturers of an ASR Versus an RUO Reagent?

Manufacturers establish and follow current Good Manufacturing Practices (cGMPs), as established in the quality system regulation, to help ensure that their products are manufactured under controlled conditions that assure the devices meet consistent specifications across lots and over time, 21 CFR Parts 808, 812, and 820. ASRs must be manufactured following cGMPs. 21 CFR 809.20. FDA does not expect RUO reagents to be manufactured in compliance with cGMPs because products labeled as RUO reagents cannot be used as clinical diagnostic products. 21 CFR 809.10(c)(2)(i). There is some controversy surrounding this and the fact that CAP regulations discourage but allow use of RUO reagents if ASR or IVD reagents are not available. CAP requires that assays developed using RUO reagents be validated by the performing laboratory, and that there be documentation of at least annual attempts to identify appropriate ASR and IVD reagents to replace the RUO reagents as they become available. Some vendors in the IHC industry have expressed concern that this "RUO loophole" in CAP guidelines promotes the use of the RUO designation by industry rather than enduring the challenge of an ASR designation.

References: [47, 67–71].

1.26 What Is the College of American Pathologists (CAP) Laboratory Accreditation Program (LAP)?

The CAP Laboratory Accreditation Program (LAP) is an internationally recognized program and the only one of its kind that utilizes teams of practicing laboratory professionals as inspectors. Designed to go well beyond regulatory compliance, the program helps laboratories achieve the highest standards of excellence to positively impact patient care.

The program is based on rigorous accreditation standards that are translated into detailed and focused checklist requirements. The checklists, which provide a quality practice blueprint for laboratories to follow, are used by the inspection teams as a guide to assess the overall management and operation of the laboratory.

The Centers for Medicare and Medicaid Services (CMS) has granted the CAP LAP deeming authority acceptable for CLIA accreditation. It is also recognized by the Joint Commission on Accreditation of Healthcare Organizations (JCAHO), and can be used to meet many state certification requirements.

More than 6,000 laboratories worldwide are accredited through CAP LAP.

CAP guidelines are constantly being updated to address changes in technology and current best practices, so the laboratory should refer to materials provided by CAP for up to date guidelines. This chapter has included some specific requirements of the CAP LAP that relate specifically to IHC and ISH to help the section supervisor to prepare for CAP inspections, and in doing so, produce best laboratory practices. (http://www.cap.org).

References: [8, 45, 76].

1.27 What Are the CAP Regulations for Content of Procedure Manuals?

Procedure manuals may be paper or electronic. Electronic manuals are easier to manage, especially in larger laboratories where the manual must be available at multiple benches so making changes will not require updating multiple paper copies. If online manuals are used, backup copies on paper or CD must be available in the case of system downtime. Procedure manuals must include step by step instructions for performance of calibration and testing procedures for each method in current use and include access to any procedures retired in past 2 years. Manuals may include procedures provided by manufacturers if they describe the actual procedure employed in the lab. Any variations from manufacturer materials would require additional documentation so that the actual procedure is documented. Acceptable specimen conditions for testing must be defined in the manual, including fixation type and time, as well as conditions that may render a specimen unacceptable, such as hemorrhage, necrosis or autolysis. The location of batch control slides must be stated in the procedure manual to be available to all pathologists working with those stains. There must be annual documentation of review of the procedure manual by director or designee and testing personnel.

Reference: [8].

1.28 What Are the CAP Regulations for Instrument and Reagents Management?

All reagents must be properly labeled including expiration date. Dates may be recorded on the containers or in a paper or electronic log providing that all containers are labeled to be traceable to the appropriate data in the log. If the manufacturer assigns an expiration date, it must be observed. If no expiration date is supplied by the manufacturer, the acceptable performance of must be determined on an annual basis. All reagents must be stored as recommended by the manufacturer. There must be documentation of proper temperatures of refrigerators used for reagent storage. There must be documentation that the pH of the buffers used in IHC is tested when a new batch is prepared or received and routinely monitored. Maintenance records of automated IHC staining instruments and validation and calibration records of digital image analysis equipment should be kept.

Reference: [8].

1.29 What Are the CAP Regulations for Microwaves Used for IHC Procedures?

Microwave devices used for heat induced epitope retrieval (HEIR) must be monitored for consistency at least annually. Reproducibility may be evaluated by monitoring the temperatures of identical samples after microwave processing. Microwave devices used for hazardous or infection materials (excluding water, certain biological stains, paraffin tissue sections) should be placed in an appropriate ventilation hood or have an integral fume extractor that is certified by the manufacturer to contain airborne chemical contaminants and potentially infectious agents. The laboratory should consult the material safety data sheets (MSDS) received with reagents and stains to assist in determining proper handling requirements and safe use. Venting containers placed in microwave devices is necessary so that processing occurs at atmospheric pressure and to prevent explosion. For procedures above atmospheric pressure, specialized containers must be used strictly by manufacturer instructions. The effectiveness of microwave ventilation should be monitored at least annually. The microwave device should be tested for radiation leakage if there is visible damage to the device.

References: [8, 77, 78].

1.30 What Are the CAP Regulations for Formaldehyde and Xylene Use?

The laboratory must have documentation of safe levels of formaldehyde and xylene vapors if used. Periodic measurements of formaldehyde and xylene vapors must be performed until results from two consecutive sampling periods taken at least 7 days apart show that employee exposure is below the action level and the short-term exposure limit (Table 1.1).

Table 1.1 Formaldehyde and xylene exposure limits

	Formaldehyde (ppm)	Xylene (ppm)
Action level (8-h time-weighted exposure)	0.5	100
15 min short-term average exposure limit (STEL)	2.0	150

Repeated measurement is required any time there is a change in production, equipment, process, personnel, or control measures, or when personnel report symptoms of respiratory or dermal conditions that may be associated with formaldehyde exposure.

References: [8, 79].

1.31 What Are the CAP Regulations for Positive Controls?

Positive controls should be performed in parallel to patient specimens and performed in the same manner and by the same personnel as patient samples. The laboratory director or designee must document the adequacy of controls, either in internal laboratory records or in the patient report each day of patient testing, and retain these records for 2 years. A statement such as, "All controls show appropriate reactivity" is sufficient. Ideal positive control tissue is present on the same slide and of the same tissue type as the patient tissue sample that possesses a low level of expression of the target antigen near the threshold of detection of the assay. Internal controls, such as normal breast ducts in hormone receptor assays, are often the best control for appropriate fixation and retrieval. Multi-tissue array blocks containing a variety of routinely processed tissue types known to both express and lack the target antigens may act as both positive and negative controls on the same slide.

An inventory of routinely processed formalin-fixed tissue samples can be used for patient specimens. These control tissues may be of different type from the patient specimen (decalcified tissues, alcohol fixed aspirate smears) if the laboratory has documented equivalent immunoreactivity by parallel testing a small panel of common markers. When batch controls are run, slides should be readily available to all pathologists working with those stains and the location of batch controls should be stated in the procedure manual. Batch controls must be reviewed by the laboratory director or designee each day of patient testing. Records of this daily review must be maintained documenting positive and negative controls for all antibodies stain appropriately. For quantitative IHC testing, control materials at more than one level may be required to verify test performance at relevant decision points. Quantitative control results must be recorded and reviewed at least monthly to evaluate trends and detect problems. Control records must be readily available to the person performing the test. Immunofluorescence assays may utilize appropriate internal positive control reactions such as IgA-positive renal tubular casts, C3 positive arterial walls. For in situ hybridization (ISH) testing, internal or external control loci should be used during each hybridization. When available, a locus-specific probe at a different site on the same chromosome and/or a normal locus on the abnormal homolog should be used. For assays that may lack an internal control locus (e.g., a Y chromosome probe in a female), an external control that is known to have the probe target should be run in parallel with the patient sample.

References: [8, 38, 39, 80].

1.32 What Are the CAP Regulations for Negative Controls?

CAP made a major change to the guidelines for the use of negative controls in 2013 now only requiring that appropriate negative controls are used. This is a recognition that modern polymer-based detection systems are sufficiently free of background reactivity that negative controls may be omitted. But if a laboratory is using biotin based detection for any assay, a negative control is still required to detect false positive staining of tissues containing endogenous biotin. Negative control sections of the patient tissue sample should be performed in parallel to patient specimens for each block tested to assess non-specific staining (specificity) related to intrinsic tissue elements (biotin or peroxidase), antigen retrieval conditions or the detection system. Appropriate staining of negative controls must be documented. The ideal negative control for monoclonal primary antibodies replaces the primary antibody with an unrelated antibody of the same isotype as the primary antibody. For polyclonal primary antibodies, an unrelated antibody from the same animal species as the primary antibody can be used. For staining kits, the negative control reagent specified by the vendor documentation and included in the kit should be used. Multi-tissue array blocks containing a variety of routinely processed tissue types known to both express and lack the target antigens may act as both positive and negative controls on the same slide. An acceptable negative control is a separate section of patient tissue processed using the same reagents and epitope retrieval protocol as the patient test slide, except that the primary antibody is omitted, and replaced by diluent/buffer solution in which the primary antibody has been diluted. When performing panels of antibodies on sections from the same block employing varied

antigen retrieval procedures, a reasonable negative control is to test the most aggressive retrieval procedure in the panel. Antigen retrieval aggressiveness (in decreasing order): pressure cooker, enzyme digestion, boiling, microwave, steamer, water bath. High pH retrieval is more aggressive than retrieval in buffer at pH 6.0. In the case of multiple blocks of similarly processed and stained sentinel lymph nodes, a single section from one of the blocks may be acceptable as the negative control reaction. Appropriate internal negative staining reactions can be considered adequate in lieu of separate negative control tissue sections. Immunofluorescence assays require separate sections of patient tissues omitting the primary antibody to act as negative control for autofluorescence.

References: [8, 38, 39, 56, 60, 72, 80].

1.33 What Are the CAP Regulations for Endogenous Biotin Blocking?

If the laboratory uses biotin in primary or dual detection systems, there must be a policy that addresses nonspecific false-positive staining from endogenous biotin. Negative controls must be performed to test for false positive staining of cell types with high metabolic activity containing abundant mitochondria with the coenzyme biotin. Hepatocytes, renal tubules, gestational endometrium and many tumors are known to be rich in endogenous biotin. False-positive staining localized to metabolically active tumor cells may occur and be easily misinterpreted. Commercial and in-house (egg whites, milk) reagents should be used to block endogenous biotin before applying the biotin-based detection systems.

References: [8, 81].

1.34 What Are the CAP Regulations for New Antibody Validation?

Validation of all antibody assays must be performed prior to use in patient diagnosis to document the performance in its proposed differential diagnostic applications. The laboratory director or qualified designee must sign a statement documenting review of validation studies and approval of each test for clinical use. A statement such as "This validation study has been reviewed, and the performance of the method is considered acceptable for patient testing" should satisfy this requirement. With the exception of prescribed validation procedures for predictive markers Her-2/neu and hormone receptors (see Chap. 9), the specific parameters of IHC validation are left to the discretion of a qualified laboratory director. General guidance is given to require testing a sufficient number of cases to provide an idea of sensitivity and specificity of the assay, and similarity of the assay to expected results. In general, a minimum of 10 positive and 10 negative tissues should be documented having appropriate results for well-established antibodies. More may be required for newer antibodies for which there is less experience in the literature. Antibodies FDA-designated as In Vitro Diagnostic (IVD) antibodies require demonstration of equivalence of staining reactions with expected results provided in the product literature supplied with the antibody from the vendor in order to validate performance in the laboratory. Vendors make no claims regarding expected performance of antibodies designated as analyte specific reagents (ASR). Therefore, validation of ASR antibodies requires establishing the sensitivity and specificity of the assay in the laboratory. The laboratory must establish or verify the performance characteristics of tests using Class I ASRs in accordance with the Method Performance Specifications section of the Laboratory General Checklist. For testing to be performed on any specimens with significantly different handling (decalcification, frozen tissues, alternative fixatives, cytologic smears), additional validation of equivalent immunoreactivity with at least small panels of samples is needed. There may be an exception to these general validation guidelines when a tumor needed for validation is too rare to make a full validation feasible.

References: [8, 38, 39, 44, 45, 56, 79].

1.35 What Are the CAP Regulations for Validation of IHC Assays When Changes Have Been Made?

Laboratories must perform a validation of IHC assay performance when conditions change that may affect performance. To confirm assay performance, testing should be performed on at least two known positive and two known negative specimens when changes are made to antibody dilution, antibody vendor of the same clone, incubation time or retrieval time. When changes are made to fixative type, retrieval method, detection system, tissue processing, laboratory location or water supply, the laboratory must confirm assay performance by testing a sufficient number of cases to ensure the assay achieves expected results. The exact number of cases required is not specifically given and is left up to the laboratory director's judgment.

Reference: [8].

1.36 What Are the CAP Regulations for Validation of New Reagent Lots?

The performance of all types of new reagent lots (enzyme, antibody, detection system) must be validated prior to use on patient tissues. Documentation of equivalent staining of serial sections from a multi-tissue control tissue block including positive and negative tissue reactions stained in parallel using old and new lots will satisfy this requirement.

References: [6, 8, 80].

1.37 What Are the CAP Regulations for Reporting IHC Results Including ASRs?

If IHC or ISH is reported as an addendum or separate procedure, there must be a mechanism to reconcile morphologic diagnosis with potentially conflicting results of special studies such as immunohistochemistry.

If the laboratory employs antibodies or nucleic acid probes designated as an Analyte Specific Reagent (ASR), federal regulations require that the following disclaimer accompany the test result on the patient report.

This test was developed and its performance characteristics determined by (laboratory name). It has not been cleared or approved by the U.S. Food and Drug Administration.

CAP recommends adding the following statement to the ASR disclaimer: The FDA has determined that such clearance or approval is not necessary. This test is used for clinical purposes. It should not be regarded as investigational or for research. This laboratory is certified under the Clinical Laboratory Improvement Amendments (CLIA) as qualified to perform high complexity clinical laboratory testing.

There is no specific guidance from CAP regarding the use of a disclaimer for "research use only" (RUO). But the laboratory may put a single ASR disclaimer on the pathology report to address all IHC and ISH studies used in a particular case. Separately tracking each reagent used for a case and selectively applying the disclaimer to only the class I ASRs is unnecessary.

CAP has additional requirements for reporting results of predictive marker studies for breast cancer which are addressed in Chap. 9.

References: [8, 46].

1.38 What Are the CAP Regulations for Slide or Slide Image Retention?

IHC slides including the control slides must be readable and retained for 10 years. Fluorescence slides will fade over time, so a diagnostic image of the fluorescent slide findings should be included on the report or maintained separately for 10 years to meet the requirement of being readable for 10 years. Representative images of FISH assays with at least one cell for normal results, and at least two cells for each abnormal result must be retained for 10 years' documentation.

References: [8, 34, 82].

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