



Principles of Analytic Validation of Immunohistochemical Assays

Frequently Asked Questions (FAQs)

1. **Why is this guideline needed? Is there any evidence that patients have been harmed by incorrect immunohistochemistry tests?**
 - a. There is ample evidence that improper immunohistochemistry (IHC) tests have led to patient harm. In perhaps the best documented example, nearly 400 of 1,000 breast cancers tested in one laboratory in Newfoundland from 1997-2005 initially classified as ER negative were subsequently found to be ER positive. Because of the incorrect test results, these patients did not receive appropriate therapy and more than 100 died. A governmental inquiry determined that the high error rate was due to improper testing practices. The American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) guideline for hormone receptor and HER2 testing in breast cancer were a direct result of well documented testing inaccuracies.
2. **How will the guideline be enforced? What happens if a laboratory doesn't follow the guideline?**
 - a. As with any clinical evidence-based guideline they are not mandatory. These recommendations *may* be incorporated into future versions of the CAP Laboratory Accreditation Program (LAP) Checklist; however, they are not currently required by LAP or any regulatory or accrediting agency. It is encouraged that laboratories adopt these evidence-based recommendations.
3. **When validating an estrogen receptor (ER) assay, must we use only breast cancers for validation tissues?**
 - a. No. Since ER is most frequently used to assess eligibility for hormonal therapy in patients with breast cancer, positive and negative breast cancers should comprise at least part of the validation set, but other ER positive and negative tissue types could be included.
4. **How do these recommendations apply to assays for pathogen-specific antigens (e.g., *Helicobacter pylori*)?**
 - a. Assays for infectious organisms are similar to predictive marker assays in that the results can directly influence patient treatment, but selection of validation sets can be quite challenging when the organism is rarely encountered. The option of using normal tissues for positive cases is also not applicable. For selected organisms, including *H. pylori*, *Cryptococcus* spp, cytomegalovirus and herpes simplex I/II, histologic features may be sufficiently characteristic to provide "expected" positive cases for validation purposes, but for true analytic validation, concurrent culture evidence of specific infection or either retrospective or prospective molecular confirmation of the formalin fixed paraffin embedded sample may be required.
5. **For rare antigens, do laboratory directors have the flexibility to use fewer validation samples as they deem appropriate?**
 - a. Yes. Following public comment and independent peer review of the draft recommendations, it was determined that the guideline should not be too prescriptive and that the medical director must have the discretion to modify the recommended steps in cases where it is not possible to gather a full validation set. Several of the final

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recommendations include the caveat that the laboratory medical director may decide that fewer cases are sufficient for a specific marker (e.g., rare antigen); however the rationale for that decision needs to be documented. If the laboratory is unable to find sufficient cases to provide reasonable confidence that test results are valid, the director is responsible for the decision to offer that test.

- 6. Are normal tissues prohibited in validation sets?**
 - a. No. Normal tissues may be used in conjunction with neoplastic and lesional tissue as appropriate, but the guideline specify that normal tissues cannot comprise the entire validation set for markers that are primarily used in diagnosing neoplasms. If the marker will be used to determine cell lineage in neoplasms, at least some of the tissues in the validation set should be neoplasms with positive and negative expression for that marker.

- 7. What is the difference between a tissue microarray (TMA) and a multitissue block (MTB)?**
 - a. The terms are not always used consistently and TMAs and MTBs are not necessarily different. TMA often refers to a tissue block constructed using a commercially available instrument that results in uniform cores while MTBs may be assembled manually; these are sometimes referred to as "sausage blocks" or "spring rolls."

- 8. If we temporarily move our laboratory while the existing one is being remodeled, do we have to revalidate all assays after both moves?**
 - a. A complete revalidation of all assays is not required when equipment is moved, but a limited assessment of a selection of assays is recommended following laboratory relocation. In this situation, re-assessment of assay performance would apply to both moves. After each move, the laboratory medical director should select a group of assays that encompass different clinical uses (i.e., predictive and non-predictive markers, pathogen-specific markers, etc) and immunolocalizations (i.e., nuclear, cytoplasmic and membranous) and compare results of testing after the move with the results of testing done previously. The number of validation tissues tested should be determined by the director.

- 9. Does the guideline address validation of research use only (RUO) antibodies?**
 - a. Not specifically, but the principles of analytic validation described in the guideline apply to all antibodies that may be used in patient testing.

- 10. Could you give some advice on the interpretation of the following terminology for IHC tests:**
 - Accuracy/Precision (Repeat measurement of samples at various concentrations or activities)
 - Sensitivity (Lower limit of detection)
 - Specificity
 - Reportable Range (Analytic Measurement Range)
 - a. CLIA requirements to determine test performance specifications apply to all lab tests including all IHC assays, but the nature of these assays is such that some of them aren't relevant. For instance, reportable range and reference intervals are generally not applicable to tests that are reported qualitatively or semi-quantitatively, which represents most IHC tests.

With respect to determining accuracy, precision, analytical sensitivity and analytical specificity, CLIA distinguishes between FDA approved and laboratory-developed tests (LDTs). For FDA-approved test kits, laboratories must demonstrate performance characteristics that are comparable to those established by the manufacturer (often

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called "verification"). Manufacturers may provide users with directions and/or materials for this verification. By contrast, laboratories must "establish" their own performance specifications for LDTs. For IHC assays, accuracy, analytic sensitivity and specificity are determined by analytic assay validation, which is theoretically done by testing a validation tissue set against a gold standard. Since the majority of IHC tests do not have a "gold standard" referent test, analytic sensitivity and specificity are determined by measuring positive and negative concordances with an appropriate comparator. This may be another validated IHC assay (i.e., different clone), testing done in another lab with a validated assay, a different test (e.g., ISH), or even clinical outcome if you have the resources. For most laboratories and tests, it's some combination of the first two.

In our literature review we could not find strong evidence to say how IHC assay precision (inter-run and inter-operator) should be measured. Until stronger evidence is available, the laboratory director must determine the extent to which these performance specifications are established based on the method, testing conditions and personnel performing the test.

- 11. Aren't commercially available antibodies already validated for clinical use by manufacturers?**
 - a. The guideline applies to analytic validation of assays, not antibodies. An antibody marketed as an FDA Class I in vitro diagnostic device may be produced following current good manufacturing practices and with documentation of specificity, but if the laboratory's assay is improperly designed or is not performed correctly (e.g. incorrect antibody dilution, inadequate antigen retrieval, wrong buffer, incorrect scoring system used), the test results will be incorrect. For antibodies marketed as "analyte specific reagents," the laboratory performing the test must establish the performance characteristics of the clinical assay.

- 12. Does the guideline apply to validation of controls?**
 - a. No. The guideline applies to assays, not antibodies or controls.

- 13. Can negative internal cells be used as a negative tissue test or do the negative validation samples need to be separate tissue samples?**
 - a. In some cases a section of tissue may contain both antigen-positive cells and negative internal control cells, and therefore serve as both a positive and negative validation challenge. When validating a new antibody lot with one positive and one negative case, for example, a single control slide that contains both antigen-positive and antigen-negative cells might be sufficient.

- 14. Does the guideline apply to assays that have been in use in the laboratory for many years or do they only apply to newly introduced assays?**
 - a. The guideline applies to all assays used on patient specimens. CLIA requires laboratories to verify the performance characteristics of all assays before issuing results on patient specimens. Thus, even if an assay has been in use, if there is no documentation that validation was ever done, the laboratory may not be compliant with federal law and could be subject to citation by an accrediting agency.

- 15. Do we have to revalidate every existing assay to provide the number of cases recommended?**
 - a. Revalidation of existing assays would not be expected if a previous validation was performed, but the Medical Director should determine if the previous validation was sufficient.

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16. Must all tissues from a validation set be acquired by and processed in the laboratory validating the IHC panel?

- a. No. This would be ideal but is not possible for many laboratories, especially reference laboratories, and may be impossible for some rare antigens.

17. How long must laboratories do validations on all the antibodies they currently use?

- a. For each assay, initial validation is done once and not repeated unless the assay is changed. Validation records should be retained indefinitely to demonstrate to future inspectors that it was done.

18. Some laboratories use microwave fixation to decrease processing time. How does this reduced fixation time influence IHC results?

- a. This specific issue was not addressed in the guideline, but because any change to a procedure can introduce variation in test results, assays done on microwave fixed tissues should be compared to routinely fixed and processed specimens to determine if IHC results are affected.

19. Is a single daily control slide sufficient for validation?

- a. No. Daily quality control is essential to ensure the assay has not changed and continues to perform as expected, but this is not a substitute for initial assay validation.