IHC Assays – New Evidence-Based Guideline for Analytic Validation

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- Past chair of CAP’s Immunohistochemistry Committee
- Member of the Center IHC Analytic Validation Principles Workgroup
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Disclosures

- Lecture Fee Paid by Entity
  - United States and Canadian Academy of Pathology (USCAP)

- Expert Witness
  - Various
Introduction

• Laboratories are required to validate all assays before testing patient specimens.

• There is significant variation in validation practices for IHC assays.

• Current guidelines exist only for HER2 and ER/PgR
Principles of Analytic Validation of Immunohistochemical Assays

Guideline From the College of American Pathologists Pathology and Laboratory Quality Center

Patrick L. Fitzgibbons, MD; Linda A. Bradley, PhD; Lisa A. Fatheree, BS, SCT(ASCP); Randa Alsaheb, MD; Regan S. Fulton, MD, PhD; Jeffrey D. Goldsmith, MD; Thomas S. Haas, DO; Rouzan G. Karabakhtsian, MD, PhD; Patti A. Loykasek, HT(ASCP); Monna J. Marolt, MD; Steven S. Shen, MD, PhD; Anthony T. Smith, MLS; Paul E. Swanson, MD

Context.—Laboratories must validate all assays before they can be used to test patient specimens, but currently there are no evidence-based guidelines regarding validation of immunohistochemical assays.

Objective.—To develop recommendations for initial analytic validation and revalidation of immunohistochemical assays.

Design.—The College of American Pathologists Pathology and Laboratory Quality Center convened a panel of pathologists and histotechnologists with expertise in immunohistochemistry to develop validation recommendations. A systematic evidence review was conducted to address key questions. Electronic searches identified 1463 publications, of which 126 met inclusion criteria and were extracted. Individual publications were graded for quality, and the key question findings for strength of evidence. Recommendations were derived from strength of evidence, open comment feedback, and expert panel consensus.

Results.—Fourteen guideline statements were established to help pathology laboratories comply with validation and revalidation requirements for immunohistochemical assays.

Recommendations were also provided for confirming assay performance when there are changes in test methods, reagents, or equipment.

(Arch Pathol Lab Med. doi: 10.5858/arpa.2013-0610-CP)
## Validation practices
### Non predictive factor assays

<table>
<thead>
<tr>
<th>Procedures</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab has written validation procedure?</td>
<td>68%</td>
<td>28%</td>
</tr>
<tr>
<td>Procedure specifies # validation cases?</td>
<td>54%</td>
<td>44%</td>
</tr>
<tr>
<td>Procedure specifies when revalidation needed?</td>
<td>46%</td>
<td>46%</td>
</tr>
<tr>
<td>Cytology specimens addressed?</td>
<td>37%</td>
<td>63%</td>
</tr>
</tbody>
</table>

Hardy et al. *Arch Pathol Lab Med* 2013;137:19-25
### Validation practices

#### Non predictive factor assays

<table>
<thead>
<tr>
<th>Procedures</th>
<th>Yes (%)</th>
<th>No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in antigen retrieval method?</td>
<td>71%</td>
<td>25%</td>
</tr>
<tr>
<td>Change in detection method?</td>
<td>74%</td>
<td>23%</td>
</tr>
<tr>
<td>Change in instrumentation?</td>
<td>74%</td>
<td>24%</td>
</tr>
<tr>
<td>Change in fixative?</td>
<td>65%</td>
<td>30%</td>
</tr>
</tbody>
</table>

Hardy et al. *Arch Pathol Lab Med* 2013;137:19-25
Introduction

• CAP convened expert and advisory panels to systematically review published data and develop evidence-based recommendations

• Closely followed IOM Clinical Practice Guidelines
  o Transparency
  o Manage conflicts of interest
  o Multidisciplinary panel
  o Patient advocate (N/A for this panel)
  o Systematic Review
  o Considered judgment
Principles of Analytic Validation for IHC Assays: Expert and Advisory Panel

**Chair**
Patrick Fitzgibbons, MD

**Expert Panel Members**
- Randa Alsabeh, MD
- Regan Fulton, MD, PhD
- Jeffrey Goldsmith, MD
- Thomas Haas, DO
- Rouzan Karabakhtsian, MD, PhD
- Patti Loykasek, HT(ASCP)QIHC
- Monna Marolt, MD
- Steven Shen, MD, PhD
- Paul Swanson, MD

**Advisory Panel Members**
- Raouf Nakhleh, MD, Center
- Richard Brown, MD
- Richard Eisen, MD
- Hadi Yaziji, MD

**Staff**
- Lisa Fathereee, SCT(ASCP)
- Tony Smith, MLS

**Consultant Methodologist**
- Linda Bradley, PhD
Systematic Evidence Review

• Identify Key Questions
• Literature search
• Data extraction
• Develop proposed recommendations
• Open comment period
• Considered judgment process
Introduction

- Overarching questions:

1. What is needed for initial analytic assay validation before placing any immunohistochemical test into clinical service?

2. What are the revalidation requirements?
1. When and how should validation assess

- analytic sensitivity
- analytic specificity
- accuracy (assay concordance)
- precision (inter-run and inter-operator variability)?
2. What is the minimum number of positive and negative cases needed to analytically validate an IHC assay for its intended use(s)?

- Non-predictive markers
- Predictive markers
- Identifying infectious organisms
- Rare antigens.

Should expression levels be specified for positive cases?
3. What parameters should be specified for the tissues used in the validation set?

- Cytology specimens
- Minimum tissue size or minimum quantity of cells
- Neoplastic vs non-neoplastic tissues
4. How do the following preanalytic variables influence analytic validation?
   - Type of fixative
   - Type of decalcification solution
   - Time in decalcification solution
   - Validation tissues processed in another laboratory

5. What conditions require assay revalidation?
Systematic Evidence Review

- Literature search
  - January 2004 – May 2013
  - 1,463 studies met inclusion criteria
    → Reviewed by panel
  - 126 studies identified for full data extraction
Systematic Evidence Review

- Evidence Evaluation
  - Quality (rate strength of evidence)
  - Quantity
  - Consistency
Quality Assessment

• Individual studies graded on specific criteria by the methodology consultant (LAB)

Criteria included

○ Quality and execution of studies

○ Quantity of data (number and size of studies)

○ Consistency and generalizability of the evidence across studies.
  - Adequate descriptions of the test
  - Adequate descriptions of the basis for the “right answer”
  - Reproducibility of test results
  - Avoidance of biases
  - Analysis of data
# Grades for Strength of Evidence

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Convincing</strong></td>
<td>Level 1 or 2 studies with an appropriate number and distribution of challenges and reported consistent and generalizable results.</td>
</tr>
<tr>
<td><strong>Adequate</strong></td>
<td>Level 1 or 2 studies that lacked the appropriate number and distribution of challenges OR were consistent but not generalizable.</td>
</tr>
<tr>
<td><strong>Inadequate</strong></td>
<td>Combinations of Level 1 or 2 studies that show unexplained inconsistencies, OR One or more lower quality studies (Level 3 or 4), OR Expert opinion.</td>
</tr>
</tbody>
</table>

Level 1: Collaborative study using a large panel of well-characterized samples; summary data from external proficiency testing schemes or inter-laboratory comparisons
Level 2: High quality peer-reviewed studies
Level 3: Lower quality peer-reviewed studies OR expert panel reviewed FDA summaries
Level 4: Unpublished or non-peer reviewed data
# Grades for Strength of Recommendation

<table>
<thead>
<tr>
<th>Designation</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strong Recommendation</strong></td>
<td>Strength of evidence is Convincing based on consistent, generalizable, good quality evidence; further studies are unlikely to change the conclusions</td>
</tr>
<tr>
<td><strong>Recommendation</strong></td>
<td>Strength of evidence is Adequate based on limitations in the quality of evidence; further studies may change the conclusions</td>
</tr>
<tr>
<td><strong>Expert Consensus Opinion</strong></td>
<td>Important validation element to address but strength of evidence is Inadequate; gaps in knowledge may require further studies</td>
</tr>
</tbody>
</table>
Systematic Evidence Review

- Open comment period (July 2013):
  - 18 draft recommendations and 5 methodology questions
  - 263 respondents; 1,037 comments
Open Comment Period

% agreement

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Systematic Evidence Review

- Considered judgment process
  - Panel reviews and considers
    - Feedback
    - Quality/quantity/consistency of evidence
    - Benefits/harms
    - Value versus cost / burdens
    - Regulatory requirements
    - Expert opinion

- 14 final recommendations
## ASCO/CAP HER2 Guideline Recommendations

### Summary of Changes

<table>
<thead>
<tr>
<th>2007</th>
<th>2013</th>
</tr>
</thead>
<tbody>
<tr>
<td>25–100 samples</td>
<td>20(+) , 20(-) for FDA-approved assays</td>
</tr>
<tr>
<td></td>
<td>40(+) , 40(-) for LDTs</td>
</tr>
<tr>
<td></td>
<td>Not applicable if assay was previously validated and lab has successful PT performance</td>
</tr>
</tbody>
</table>

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### ASCO/CAP HER2 Guideline Recommendations
#### Summary of Changes

**Concordance**

<table>
<thead>
<tr>
<th>2007</th>
<th>2013</th>
</tr>
</thead>
<tbody>
<tr>
<td>If &lt;95% for any result category, cases with that test result must be automatically reflexed to alternative method</td>
<td>Specific concordance requirements are not required Laboratories must comply with accreditation and PT requirements</td>
</tr>
</tbody>
</table>
The Guidelines
Guideline 1

Recommendation: Laboratories must validate all immunohistochemical tests before placing into clinical service.

- **Note:** Such means include (but are not necessarily limited to):
  - Correlating the new test’s results with the morphology and expected results;
  - Comparing the new test’s results with the results of prior testing of the same tissues with a validated assay in the same laboratory;
  - Comparing the new test’s results with the results of testing the same tissue validation set in another laboratory using a validated assay;
Guideline 1

Recommendation: Laboratories must validate all immunohistochemical tests before placing into clinical service.

- Note: Such means include (but are not necessarily limited to):
  - Comparing the new test’s results with previously validated non-immunohistochemical tests; or
  - Testing previously graded tissue challenges from a formal proficiency testing program (if available) and comparing the results with the graded responses.
Guideline 1

• Strength of Evidence:
  o Adequate to support when analytic validation should be done and that it should include determination of concordance and precision
  o Inadequate to assess how validation should be done with regard to the listed approaches, but did show that these approaches have been used.

• Rationale: Analytic validation provides a net benefit for the overall performance and safety of IHC tests by contributing to the avoidance of potential harms related to analytic false positive and false negative test results.
Rationale 1

• Validation set should include:
  o Positive, negative, and low positive tissues
  o Should not be all normal tissues
  o Should reflect the intended use of the assay

• Positive and negative cell types on the same section could be used as separate challenges
Guideline 2

Recommendation: For initial validation of every assay used clinically (with the exception of HER2, ER and PgR, for which established validation guidelines already exist), laboratories should achieve at least 90% overall concordance between the new test and the comparator test or expected results. If concordance is less than 90%, laboratories need to investigate the cause of low concordance.
Guideline 2

• **Strength of evidence**
  
  o **Adequate to support a 90% (versus 95%) overall concordance benchmark for analytic validation of IHC tests (except HER2, ER, PgR)**

  - Median overall concordance in a two-year inter-laboratory comparison of CD117 IHC and target results was 87.6% (Hsi, 2001)

  - Median overall concordance in 5 comparisons of different HER2 IHC tests was 89.0% (range 74–92%), with 2 of 5 studies >90% concordant. (Boers, 2011; Mayr, 2009; Moelans, 2010; O’Grady, 2010; van der Vegt, 2009)

  - Median overall concordance in 5 comparisons of HER2 IHC tests to HER2 ISH tests was 88.2% (range 66–94%), with 2 of 5 comparisons >90% concordant (Dorfman, 2006; Jordan, 2012; Lotan, 2011; Phillips 2007)

  - Median overall concordance in 6 comparisons of IHC tests (PTEN, ER, PR, HER2, MPT64, p16) to alternative referent tests (e.g., RNA expression, clinical diagnosis) was 91.4% (range 74–99%), with 3 of 6 studies >90% concordant (Phillips, 2007; Baba, 2008, Lehmann-Che, 2011)
Guideline 3

Expert Consensus Opinion: For initial analytic validation of non-predictive factor assays, laboratories should test a minimum of 10 positive and 10 negative tissues. When the laboratory medical director determines that fewer than 20 validation cases are sufficient for a specific marker (e.g., rare antigen), the rationale for that decision needs to be documented.

- Note: The validation set should include high and low expressors for positive cases when appropriate, and should span the expected range of clinical results (expression levels) for markers that are reported quantitatively.
Guideline 3

• **Strength of Evidence**
  
  o Inadequate to support the recommended number of validation samples.
  
  o Adequate to support the distinction between non-predictive and predictive IHC tests and the use of different numbers.
Validation Using 10 and 20 Tissue Validation Sets against a 90% Concordance Benchmark

<table>
<thead>
<tr>
<th># of validation tissues</th>
<th>0 discordant (95% CI)</th>
<th>1 discordant (95% CI)</th>
<th>2 discordant (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>100% (68-100)</td>
<td>90% (57-100)</td>
<td>80% (48-95)</td>
</tr>
<tr>
<td>20</td>
<td>100% (81-100)</td>
<td>95% (75-100)</td>
<td>90% (69-98)</td>
</tr>
</tbody>
</table>

Concordance estimates with 95% confidence intervals stratified by number of observed discordant samples.
Guideline 4

**Expert Consensus Opinion:** For initial analytic validation of all laboratory-developed predictive marker assays, laboratories should test a minimum of 20 positive and 20 negative tissues. When the laboratory medical director determines that fewer than 40 validation tissues are sufficient for a specific marker, the rationale for that decision needs to be documented.

- **Note:** Positive cases in the validation set should span the expected range of clinical results (expression levels). This recommendation does not apply to any marker for which a separate validation guideline already exists.
Guideline 4

• Strength of Evidence
  o Inadequate to support the recommended number of validation samples.
  o Adequate to support the distinction between non-predictive and predictive IHC tests and the use of different numbers.
Validation Using a 40 Tissue Validation Set (20 Positive and 20 Negative) against a 90% Concordance Benchmark

<table>
<thead>
<tr>
<th># of validation tissues</th>
<th>Concordance estimate (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 discordant</td>
</tr>
<tr>
<td>20</td>
<td>100% (81-100)</td>
</tr>
<tr>
<td>40</td>
<td>100% (90-100)</td>
</tr>
</tbody>
</table>

Concordance estimates with 95% confidence intervals stratified by number of observed discordant samples.
A 2x2 contingency table of a 40 tissue validation set that did not meet the benchmark (results entered into a 2x2 contingency table) with associated statistical tests.

<table>
<thead>
<tr>
<th>New IHC Result</th>
<th>Referent Result Positive</th>
<th>Referent Result Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

Overall concordance: 35/40 = 87.5% (does not meet 90% benchmark)
Kappa: 0.75
McNemar's p: 0.13, not significant
Positive concordance: 15/20 = 75%
Negative concordance: 20/20 = 100%
Guideline 5

**Recommendation:** For a marker with both predictive and non-predictive applications, laboratories should validate it as a predictive marker if it is used as such.

- **Strength of evidence:**
  - Adequate to support the use of the higher validation standard (e.g., number of samples) in the case of a marker with both non-predictive and predictive intended uses.
Guideline 6

Recommendation: When possible, laboratories should use validation tissues that have been processed using the same fixative and processing methods as cases that will be tested clinically.

• Strength of evidence
  o Adequate to support that laboratories should, whenever possible, use the same fixative and processing methods as cases tested clinically, in order to validate using representative specimens.
Guideline 6

- Can be difficult in reference laboratories that receive tissues with disparate fixation protocols
- Focused validation with a small number of markers may be appropriate
Guideline 7

*Expert Consensus Opinion:* If IHC is regularly done on cytologic specimens that are not processed in the same manner as the tissues used for assay validation (e.g., alcohol-fixed cell blocks, air-dried smears, formalin post-fixed specimens), laboratories should test a sufficient number of such cases to ensure that assays consistently achieve expected results. The laboratory medical director is responsible for determining the number of positive and negative cases and the number of predictive and non-predictive markers to test.
Guideline 7 Continued

• Strength of evidence
  o Inadequate to address the criteria and number of samples needed for validation with cytology specimens.

• Focused validation on representative antibodies used on cytologic specimens would be appropriate

• A disclaimer in the report (especially in the case of negative results) may be appropriate if assays cannot be feasibly validated:
  o “Immunohistochemistry on cytologic specimens has not been sufficiently validated; these results should be interpreted with caution.”
Guideline 8

**Expert Consensus Opinion:** If IHC is regularly done on decalcified tissues, laboratories should test a sufficient number of such tissues to ensure that assays consistently achieve expected results. The laboratory medical director is responsible for determining the number of positive and negative tissues and the number of predictive and non-predictive markers to test.
Guideline 8 Continued

• Strength of evidence:
  o Inadequate to address the criteria and number of samples needed for validation with decalcified specimens.

•Focused validation on representative antibodies used on decalcified specimens would be appropriate.

•A disclaimer in the report (especially in the case of negative results) may be appropriate if assays cannot be feasibly validated (ANP.22985).
Guideline 9

Recommendation: Laboratories may use whole sections, tissue microarrays (TMAs) and/or multitissue blocks (MTBs) in their validation sets as appropriate. Whole sections should be used if TMAs/MTBs are not appropriate for the targeted antigen or if the laboratory medical director cannot confirm that the fixation and processing of TMAs/MTBs is similar to clinical specimens.
Guideline 9 Continued

• Strength of evidence
  o Adequate to support TMA usage; however there are many variables to be considered and thorough validation is needed for each marker.
  o Inadequate to recommend the routine use of TMA samples.

• TMAs / MTBs can be very useful in many circumstances. Beware of:
  o Proteins with high levels of heterogeneity (gastric Her2)
  o Limited tissue expression (e.g. bcl-6)
Revalidation Secondary to Assay Modification

Antibody Specific

1. Least:
   - New antibody Lot

2. Moderate:
   - Antibody dilution
   - Antibody vendor (same clone)
   - Antibody incubation or antigen retrieval times (same A.R. method)

3. Most:
   - New antibody clone

All Assays (one tier):

- Fixative type
- Antigen retrieval method
  - pH change
  - buffer type
  - heat type
- Antigen detection system
- Tissue processing equipment
- Environmental conditions
  - location
  - water supply
Evidence for Revalidation Guidelines 10-13

- Strength of evidence
  - Inadequate to address conditions requiring assay revalidation and whether revalidation should be the same as initial validation.
Expert Consensus Opinion: When a new reagent lot is placed into clinical service for an existing validated assay, laboratories should confirm the assay’s performance with at least 1 known positive case and 1 known negative case.

- Laboratories may want to include low-expressors, especially with predictive markers
Guideline 11

*Expert Consensus Opinion:* Laboratories should confirm assay performance with at least 2 known positive and 2 known negative cases when an existing validated assay has changed in any one of the following ways:

- Antibody dilution
- Antibody vendor (same clone)
- Incubation or retrieval times (same method)

• Laboratories may want to include low-expressors, especially with predictive markers
**Expert Consensus Opinion:** Laboratories should confirm assay performance by testing a sufficient number of cases to ensure that assays consistently achieve expected results when any of the following have changed:

- Fixative type
- Antigen retrieval method (e.g., change in pH, different buffer, different heat platform)
- Antigen detection system
- Tissue processing or testing equipment
- Environmental conditions of testing (e.g., laboratory relocation)
- Laboratory water supply
Guideline 12 Continued

• The laboratory medical director is responsible for determining how many predictive and non-predictive markers and how many positive and negative tissues to test.

  o Reasonable approach:
    - Selection of antibodies from menu with:
      - Variable clinical uses (predictive and non-predictive)
      - Variable antigen localizations
      - Variable antibody types (monoclonal / polyclonal, etc.)
Guideline 13

*Expert Consensus Opinion:* Laboratories should run a full revalidation (equivalent to initial analytic validation) when the antibody clone is changed for an existing validated assay.
Guideline 14

Expert Consensus Opinion: The laboratory must document all validations and verifications in compliance with regulatory and accreditation requirements.
Summary

• Physicians and patients rely on accurate diagnostic and prognostic testing in the clinical laboratory.

• Analytic validation is essential to ensuring that an assay performs as expected, accurately identifies and/or quantifies the targeted analyte, and minimizes the chances of false positive or false negative results.

• Established guidelines are important to improve the reproducibility and consistency of the test results.
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  — Emerging Concepts in the Workup of Polycythemia and Thrombocythemia: JAK2

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