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Objective

Introduction

- **In 2014, the Pathology and Laboratory Quality Center for Evidence-based Guidelines (The Center) published a clinical practice guideline on analytical validation of clinical immunohistochemistry (IHC) assays.[1]**

- **Since the publication of the original guideline the landscape of clinical IHC has substantially changed with the introduction of new predictive markers and the advent of companion/complementary diagnostics.**

Principles of Analytic Validation of Immunohistochemical Assays

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

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Objective

- **The objective of the guideline update was to:**

    o **Harmonize previously variable recommendations for analytic validation/verification of predictive markers, including human epidermal growth receptor 2 (HER2), estrogen receptor (ER), and progesterone receptor (PR) IHC performed on breast carcinoma.**

    o **To create validation recommendations for companion/complementary IHC assays with distinct scoring systems based on tumor type (eg, programmed death receptor-1 [PD-L1]).**

    o **To re-evaluate the validation requirements for non-formalin fixed tissues, including cytology specimens.**

Key Questions and Results

Scope

- **Scope: The purpose of this update was to assess evidence published since the release of the original guideline to provide recommendations on how to analytically validate/verify immunohistochemical assays used for diagnostic and predictive purposes.**

- **Validation of image analysis assisted IHC interpretation is not within the scope of this document and was not covered in the update.**

- **Target Audience: Laboratory directors, Pathologists, Histology/Cytology Technicians/Technologists, and Medical Professionals involved in laboratory quality.**

Key Questions (KQs)

- **Overarching Question: What is needed for initial analytic assay validation/verification before placing any IHC test into clinical service?**

- **KQ1: For the initial validation of an assay used clinically, what is the minimum overall analytic accuracy?**

- **KQ 2: What is the minimum number of positive and negative cases that need to be tested to analytically validate immunohistochemical nonpredictive marker assays, United States Food and Drug Administration (FDA) approved/cleared predictive marker assays (including companion/complementary diagnostics), and laboratory developed predictive marker assays, for their intended use?**

Key Questions, continued

- **KQ 3: What parameters should be specified for the tissues used in the validation set?**

  - What tissue/tumor types are appropriate for inclusion in a validation set?

- **KQ 4: How do decalcification and non-formalin fixation methods (including those utilized on cytology specimens) influence analytic validation?**

- **KQ 5: What conditions require assay revalidation?**

Results

- Two strong recommendations, one conditional recommendation, and 12 good practice statements are offered in the updated guideline.
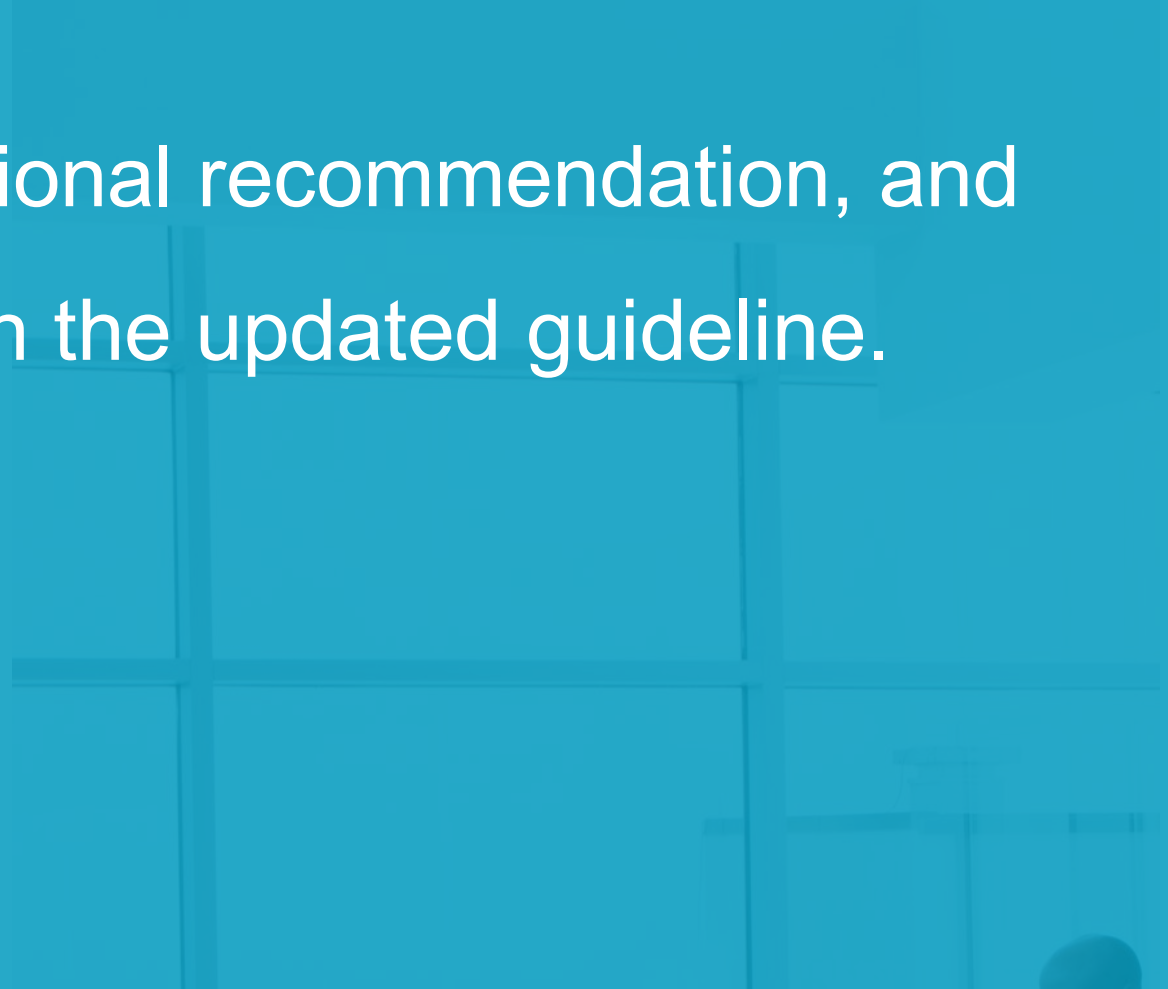
Guideline Recommendations and Good Practice Statements

Statement 1

- **Laboratories must analytically validate all laboratory developed immunohistochemistry (IHC) assays and verify all FDA-cleared IHC assays before reporting results on patient tissues. (Good Practice Statement)**

  Note: **A validation study design may include but is not necessarily limited to, such means as:**

  - Comparing the new assay's results with the expected architectural and subcellular localization of the antigen
  - Comparing the new assay's results with the results of prior testing of the same tissues with a validated/verified assay in the same laboratory
  - Comparing the new assay's results with the results of testing the same tissues in another laboratory using a validated/verified assay
  - Comparing the new assay's results with results of a non-immunohistochemical method
  - Comparing the new assay's results with the results from testing the same tissues in a laboratory that performed testing for a clinical trial
  - Comparing the new assay's results against percent positive rates documented in published clinical trials
  - Comparing the new assay's results to IHC results from cell lines that contain known amounts of protein
  - Comparing previously graded tissue challenges from a formal proficiency testing program (if available) with the graded responses

Discussion/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.**

- **CLIA (section 493.1253) requirement is for all laboratories to validate the performance characteristics of all assays used in patient testing in order to ensure that the results are accurate and reproducible.[2]**

- **Most IHC assays do not have a readily available reference standard from which analytic sensitivity and specificity can be calculated. As such, concordance with results from a comparator assay must be utilized.**

Discussion/Rationale, continued

- **Laboratory medical director has control over the design and performance of the validation/verification plan.**

- **The validation set should include:**

    o **Positive, negative, and low-positive tissues that are tailored to the intended clinical use of the assay.**

    o **Should not be all normal tissues.**

    o **Positive and negative cells on the same section could be scored as separate challenges.**

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Statement 2

- **For initial analytic validation/verification of every assay used clinically, laboratories should achieve at least 90% overall concordance between the new assay and the comparator assay or expected results.**

  (Strong Recommendation; Certainty of Evidence: Moderate)

Discussion/Rationale

- **This recommendations serves to harmonize validation requirements for all predictive markers.**

  - The guideline update applies to ER, PR, and HER2, superseding the previously different concordance thresholds.

  - Since publication of the original guideline, substantial new literature did not exist; as such, this recommendation is unchanged.

Statement 3

- **For initial analytic validation of nonpredictive laboratory developed 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 (eg, rare antigen), the rationale for that decision needs to be documented.**

  (Good Practice Statement)

  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 using either a semiquantitative or numerical scoring system.**

Statement 4

- **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.**

    (Good Practice Statement)

    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 using either a semiquantitative or numerical scoring system.**

Statement 5

- **For initial analytic verification of all unmodified FDA-approved predictive marker assays, laboratories should follow the specific instructions provided by the manufacturer. If the package insert does not delineate specific instructions for assay verification, the laboratory should test a minimum of 20 positive and 20 negative tissues. When the laboratory medical director determines that fewer than 40 verification tissues are sufficient for a specific marker, the rationale for that decision needs to be documented.**

(Good Practice Statement)

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 using either a semiquantitative or numerical scoring system.**

Discussion/Rationale

- **Predictive markers provide clinical information that is often independent of the histologic impression and may drive clinical decision-making. As such, validation/verification of these markers requires more stringent validation/verification recommendations compared with nonpredictive assays.**

- **For verification of unmodified FDA approved/cleared assays in which the package insert does not clearly state verification procedures, laboratories should test a minimum of 20 positive and 20 negative cases.**
  - **This number was selected as a vast majority of FDA approved/cleared assays are classified as predictive markers.**

Discussion/Rationale, continued

# of validation tissues	Concordance estimate (95% CI) [1]		
	0 discordant	1 discordant	2 discordant
10	100% (68-100)	90% (57-100)	80% (48-95)
20	100% (81-100)	95% (75-100)	90% (69-98)

# of validation tissues	Concordance estimate (95% CI)				
	0 discordant	1 discordant	2 discordant	3 discordant	4 discordant
20	100% (81-100)	95% (75-100)	90% (69-98)	85% (63-96)	80% (58-92)
40	100% (90-100)	97.5% (86-100)	95% (83-99)	92.5% (79-98)	90% (76-97)

Statement 6

- **For initial analytic validation of laboratory developed assays and verification of FDA-approved/cleared predictive immunohistochemical assays with distinct scoring schemes (eg, HER2, PD-L1), laboratories should separately validate/verify each assay-scoring system combination with a minimum of 20 positive and 20 negative tissues. The set should include challenges based on the intended clinical use of the assay.**

(Strong Recommendation; Certainty of Evidence: Moderate)

Discussion/Rationale

- **This recommendation pertains to predictive marker assays such as HER2 and PD-L1, where more than one scoring system exists. The applicable scoring system is determined by the tumor type and/or site of origin.**

- **This new approach acknowledges the conceptual separation between IHC readout (determination of the intensity, extent, quality, and cellular localization of immunohistochemical signal) and interpretation; the readout and associated staining protocol should be validated as a single unit.**

  o **For example, PD-L1 clone 22C3 using tumor proportion score and PD-L1 clone 22C3 using the combined positive score should be separately validated.**

  o **It is NOT the intention of this recommendation to mandate separate validations for assay/clone/tumor type combinations.**

  o **Since these are predictive markers, a minimum of 20 positive and 20 negative cases should be used.**

Statement 7

- **For laboratory developed assays with both predictive and nonpredictive applications using the same scoring criteria, laboratories should treat these assays as predictive markers and test a minimum of 20 positive and 20 negative cases.**

  (Good Practice Statement)

  Note: **See Statement 4 for additional information.**

Statement 8

- **Laboratories should use validation tissues that have been processed using the same fixative and processing methods as cases that will be tested clinically, when possible.**

  (Good Practice Statement)

Statement 9

- **For analytic validation of IHC performed on cytologic specimens that are not fixed in the same manner as the tissues used for initial assay validation, laboratories should perform separate validations for every new analyte and corresponding fixation method before placing them into clinical service. (Conditional Recommendation; Certainty of Evidence: Moderate)**

  Note: **Such cytologic specimens include (but are not necessarily limited to):**

  - o **air-dried and/or alcohol-fixed smears**
  - o **liquid based cytology preparations**
  - o **alcohol-fixed cell blocks**
  - o **specimens collected in alcohol or alternative fixative media that are post-fixed in formalin**

Discussion/Rationale

- **This new recommendation is predicated on the observation that different fixatives may impact the sensitivity of the assay. As such, tissues exposed to fixatives other than that used for initial assay validation should be separately validated/verified.**

  o **Example: if the initial assay validation is performed on FFPE histologic tissues, then any specimen that is not collected directly into formalin for fixation and processed in a manner similar to histologic tissue would require a separate validation prior to clinical use.**

  o **Examples of some specimens that recommendation would apply to include (i) direct smears and cytospin preparations that are air-dried and/or alcohol-fixed; (ii) liquid-based cytology preparations (eg, ThinPrep, SurePath); (iii) cell block preparations that use alcohol-based fixatives (eg, Cellient); (iv) specimens collected in transport media such as saline or Rosewell Park Memorial Institute (RPMI) medium or in alcohol-based fixatives (eg, CytoLyt) that are subsequently processed in formalin to create a FFPE cell block.**

Discussion/Rationale, continued

- **This recommendation is relevant for only new analytes that will be placed into clinical service and does not recommend retrospective validation of all antibodies that have been previously validated and currently in use on cytologic specimens.**

Statement 10

- **A minimum of 10 positive and 10 negative cases is recommended for each validation performed on cytologic specimens, if possible. The medical director should consider increasing the number of cases if predictive markers are being validated. If the minimum of 10 positive and 10 negative cases is not feasible, the rationale for using fewer cases should be documented.**

(Good Practice Statement)

Statement 11

- **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 nonpredictive markers to test.**

(Good Practice Statement)

Discussion/Rationale

- **A focused validation on representative antibodies used on decalcified specimens (eg, bone marrow biopsies) would be appropriate.**

- **A disclaimer on the report (especially in the case of negative results) may be appropriate if assays cannot be feasibly validated (LAP checklist ANP.22985).[3]**

Statement 12

- **Laboratories should confirm assay performance with at least 1 known positive and 1 known negative tissue when a new antibody lot is placed into clinical service for an existing validated assay (a control tissue with known positive and negative cells is sufficient for this purpose).**

(Good Practice Statement)

Statement 13

- **Laboratories should confirm assay performance with at least two known positive and two known negative tissues 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)**

(Good Practice Statement)

Statement 14

- **Laboratories should confirm assay performance by testing a sufficient number of tissues to ensure that assays consistently achieve expected results when any of the following have changed:**

  o **Fixative type**

  o **Antigen retrieval method (eg, change in pH, different buffer, different heat platform)**

  o **Detection system**

  o **Tissue processing equipment**

  o **Automated testing platform**

  o **Environmental conditions of testing (eg, laboratory relocation, laboratory water supply)**

  (Good Practice Statement)

Statement 15

- **Laboratories should run a full revalidation (equivalent to initial analytic validation) when the antibody clone is changed for an existing validated assay.**

  (Good Practice Statement)

Discussion/Rationale

- **Modifications to the assay conditions may affect results. As such, laboratory medical directors should verify assay performance after a change in conditions.**

Guideline Development Process

Expert Panel (EP)

- **Jeffrey D. Goldsmith, MD***

- **Megan L. Troxell, MD, PhD**

- **Sinchita Roy-Chowdhuri, MD, PhD**

- **Mary Elizabeth Edgerton, MD, PhD**

- **Patrick L. Fitzgibbons, MD**

- **Regan Fulton, MD, PhD**

- **Thomas Haas, DO**

- **Patricia L. Kandalaft, MD**

- **Patti Loykasek, HTL(ASCP)**

- **Paul E. Swanson, MD**

- **Andrew Michael Bellizzi, MD**

- **Carol F. Colasacco, MLIS, SCT(ASCP)▪**

- **Tanja Kalicanin, MLS(ASCP)ᶜᵐ▪**

- **Christina Lacchetti, MHS**

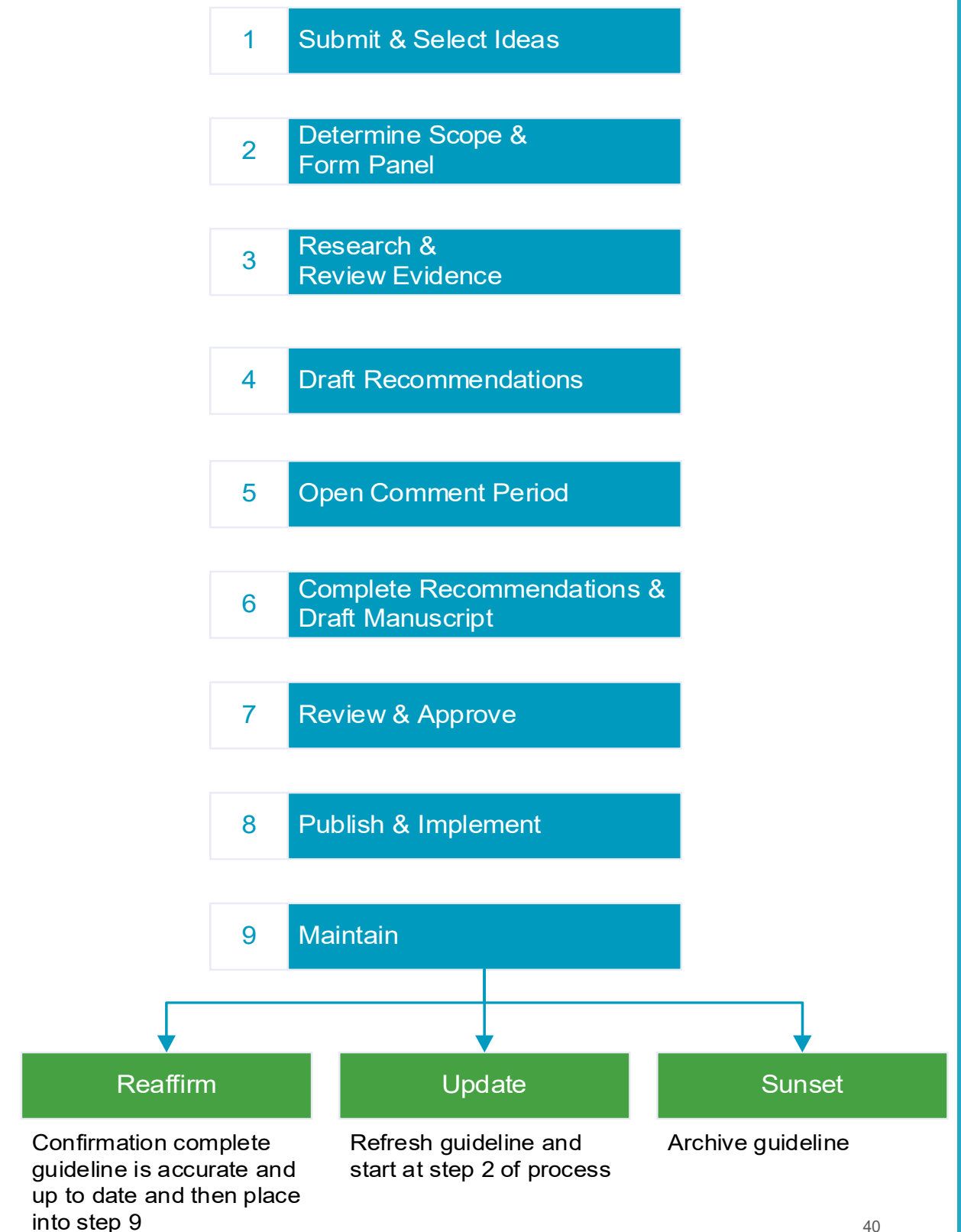- **Nicole E. Thomas, MPH, CT(ASCP)ᶜᵐ▪**

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- **Richard N. Eisen, MD**

- **Rouzan Karabakhtsian, MD, PhD**

- **Homa Keshavarz, PhD**

- **Chad Livasy, MD**

- **David Rimm, MD, PhD**

- **Lori Schmitt, HT(ASCP) QIHC**

- **Robert Schwartz, MD**

- **Thomas Summers, MD, MBA**

Guideline Development Process

- **The Center follows the standards endorsed by the National Academy of Medicine for developing Clinical Practice Guidelines.**

- **Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach was utilized in updating the guideline.**

- **A detailed description of the guideline development process can be found online**
  Evidence-based Guidelines Development Methodology Manual

1	Submit & Select Ideas
2	Determine Scope & Form Panel
3	Research & Review Evidence
4	Draft Recommendations
5	Open Comment Period
6	Complete Recommendations & Draft Manuscript
7	Review & Approve
8	Publish & Implement
9	Maintain

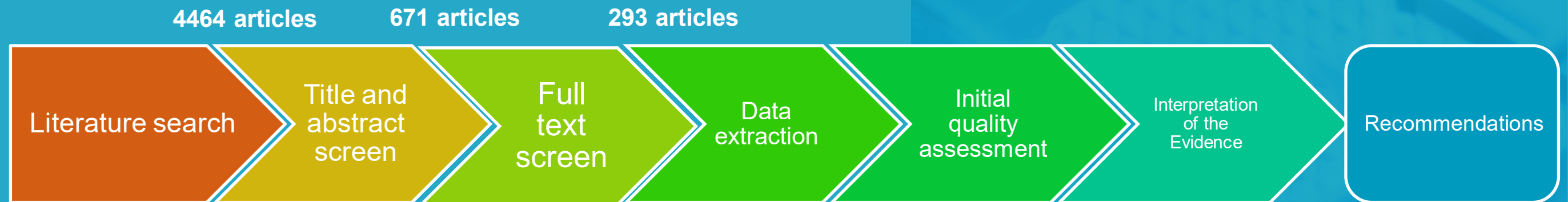| Reaffirm	Update	Sunset
Confirmation complete guideline is accurate and up to date and then place into step 9	Refresh guideline and start at step 2 of process	Archive guideline

Literature Search

- **Search was conducted in Ovid MEDLINE, Embase, Cochrane Library.**

- **Initial literature search ran on: 4/9/2019**
  - **To capture articles from 1/1/2012 – 4/9/2019)**
  - **A total of 2447 studies were captured**

- **Literature refresh on 8/4/2021**
  - **To capture articles from 4/9/2019 – 8/4/2021**
  - **A total of 1402 studies were captured**

- **Second literature refresh on 10/24/2022**
  - **To capture articles from 8/4/2021 – 10/24/2022**
  - **A total of 615 studies were captured**

Systematic Review of the Literature

- **Each level of systematic review (title-abstract screening, full-text review, and data extraction) was performed in duplicate by two members of the expert panel.**

4464 articles　　**671 articles**　　**293 articles**

Literature search → Title and abstract screen → Full text screen → Data extraction → Initial quality assessment → Interpretation of the Evidence → Recommendations

Quality Assessment

- **Systematic Reviews (SRs) and Meta-analyses questions were assessed as per the Assessing the Methodological Quality of SRs (AMSTAR) tool.[4]**

- **Non-randomized studies were assessed using the Risk of Bias in Non-Randomized Studies – of Intervention (ROBINS-I) tool.[5]**

- **Diagnostic studies were assessed using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) tool.[6]**

Open Comment Period

- **Open Comment Period held from August 4, 2021 to August 25, 2021**

- **Results**

  o **More than 350 written comments were received**

  o **Seven draft statements achieved more than 90% agreement, 6 statements received between 80%-90%, and 1 draft statement received below the 80% agreement**

Review and Approval

- **The AP reviewed and provided feedback on the draft recommendations and manuscript.**

- **The EP approved the final recommendations and good practice statements with a formal vote.**

- **The independent review panel (IRP) representing the Council on Scientific Affairs reviewed and approved the guideline for the CAP.**

  o **IRP members were masked to the expert panel and vetted through the conflicts of interest (COI) process**

Conclusion

Conclusion

- **In summary, the guideline update provides additional recommendations regarding validation of IHC assays performed on cytology specimens and predictive marker assays that have distinct scoring systems.**

- **Absence of calibrated reagents against which new IHC assays can be compared is a major limitation in the IHC validation space. Such products are critical for future standardization of clinical IHC assays.**

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2. US Department of Health and Human Services. Clinical laboratory improvement amendments of 1988: Final rule: *Fed Regist*; 1992;57(40):7001-7186. Codified at 42 CFR §1405-1494.

3. College of American Pathologists. Accreditation Checklists. College of American Pathologists website. Updated 2023. Accessed December 28, 2028. https://www.cap.org/laboratory-improvement/accreditation/accreditation-checklists.

4. Shea BJ, Grimshaw JM, Wells GA, et al. Development of AMSTAR: A measurement tool to assess the methodological quality of systematic reviews. *BMC Med Res Methodol.* 2007;7:10. doi:10.1186/1471-2288-7-10

5. Sterne JA, Hernan MA, Reeves BC, et al. ROBINS-I: A tool for assessing risk of bias in non-randomised studies of interventions. *BMJ*. 2016;355:i4919. doi: 10.1136/bmj.i4919

6. Whiting PF, Rutjes AW, Westwood ME et al. QUADAS-2: A revised tool for the quality assessment of diagnostic accuracy studies. *Ann Intern Med*. 2011;155(8):529-36. doi:10.7326/0003-4819-155-8-201110180-00009

7. Goldsmith JD, Troxell ML, Roy-Chowdhuri S, et al. Principles of analytic validation of immunohistochemical assays: guideline update. *Arch Pathol Lab Med*. Published online February 23, 2024. doi: 10.5858/arpa.2023-0483-CP

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- **The CAP developed the Pathology and Laboratory Quality Center for Evidence-based Guidelines as a forum to create and maintain laboratory practice guidelines (LPGs). Guidelines are intended to assist physicians and patients in clinical decision-making and to identify questions and settings for further research. With the rapid flow of scientific information, new evidence may emerge between the time an LPG is developed and when it is published or read. LPGs are not continually updated and may not reflect the most recent evidence. LPGs address only the topics specifically identified therein and are not applicable to other interventions, diseases, or stages of diseases. Furthermore, guidelines cannot account for individual variation among patients and cannot be considered inclusive of all proper methods of care or exclusive of other treatments. It is the responsibility of the treating physician or other health care provider, relying on independent experience and knowledge, to determine the best course of treatment for the patient. Accordingly, adherence to any LPG is voluntary, with the ultimate determination regarding its application to be made by the physician in light of each patient's individual circumstances and preferences. CAP makes no warranty, express or implied, regarding LPGs and specifically excludes any warranties of merchantability and fitness for a particular use or purpose. CAP assumes no responsibility for any injury or damage to persons or property arising out of or related to any use of this statement or for any errors or omissions.**

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