



Molecular Diagnostic Assay Validation

Update to the 2009 AMP Molecular Diagnostic Assay Validation White Paper

Members of the 2013 and 2014 Clinical Practice Committees of the Association for Molecular Pathology

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Standard of practice is not defined by this article, and there may be alternatives. See Disclaimer for further details.

INTRODUCTION

The goal of method validation in the molecular diagnostics laboratory is to ensure that a given test is ready for implementation in the clinical laboratory. To reach that goal, each step of the testing process must be carefully evaluated and documented. Such validation is relatively standardized for high volume automated assays in fields such as clinical chemistry. It is challenging to apply those standardized practices to molecular diagnostics laboratories, which rely heavily on low-volume labor-intensive tests, both FDA approved and laboratory-developed. Excellent and comprehensive documents have recently been published on this topic¹⁻¹⁰. This overview is intended to provide a brief practical reference that can be used to guide the validation process in molecular diagnostics laboratories. In addition, a sample summary checklist is provided in the appendix as a template to facilitate documentation of laboratory director review and sign off on the validation process when new assays are developed or adopted.

SUMMARY

CLIA (42 CFR 493.1253) and CAP (MOL 30785-31705) require that laboratories validate the performance of tests before reporting patient results.

DEFINITIONS

Assay Validation

- *WHO definition*: “the action (or process) of proving that a procedure, process, system equipment or method used works as expected and achieves the intended result”¹¹.
- *FDA definition*: “confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use can be consistently fulfilled”¹².
- *CLSI definition*: “confirmation by examination and provision of objective evidence through a defined process that the particular requirements for a specific intended use can be consistently fulfilled.”¹³ This definition is also used by CAP Accreditation Programs¹⁴.

Assay Verification

- *CLIA definition*: “confirmation by examination and provision of objective evidence that specified requirements have been fulfilled”¹³, or “confirmation that the laboratory using a test can replicate the manufacturer’s performance claims when the test is used according to the package insert”¹⁵.
- *CAP definition*: “an abbreviated process to demonstrate that a test performs in substantial compliance to previously established claims”¹⁴

Verification applies to unmodified non-waived (moderate- and high-complexity) tests that have been cleared or approved by the FDA and are labeled “for *in vitro* diagnostic use.”

ASSAY TYPES

UNMODIFIED FDA-Approved or Cleared Tests – Requirements

For UNMODIFIED FDA-approved or FDA-cleared tests, laboratories must **verify** that test(s) perform(s) as expected by obtaining data on:

- Accuracy
- Precision
- Reportable range
- Linear range (for quantitative assays)
- Reference intervals (normal values) for laboratory patient population

These performance characteristics are published in the manufacturer’s package insert.

MODIFIED FDA-Approved or non-FDA Cleared Tests – Requirements

For MODIFIED FDA-Approved tests or for non-FDA cleared tests (e.g., Laboratory Developed Procedures (LDP), previously LDTs) laboratories must **establish** the following performance characteristics:

- Accuracy
- Precision
- Reportable range
- Linear range (for quantitative assays)
- Reference intervals (normal values) for laboratory patient population
- Analytic sensitivity
- Analytic specificity

For some tests there may be additional parameters which are necessary to assess:

- Frequency or call rate for genotyping assays
- Specimen stability
- Carryover (e.g., well-to-well cross-contamination for automated nucleic acid extraction)

ASSAY DESIGN

- Define the requirements of the test (intended use, test method and expected performance characteristics (e.g. accuracy, precision, reportable range, analytical sensitivity, analytical specificity, reference interval)
- Review the literature to support evidence for clinical utility and clinical validity of the test
- Assess clinical indication for the test
- Define target population
- Define purpose of the test (e.g., screening, diagnosis, prognosis, monitoring)
- Choose pertinent specimen types
- Establish criteria for sample rejection (e.g., sample age and quantity, preferred anticoagulant for collection tubes)

- Establish minimal acceptance performance criteria of the test (e.g., test turnaround time, coefficient of variation of the assay)
- Consider the role of test result in patient management
- Assess technical feasibility of assay implementation (e.g., right equipment, manpower, enough samples to justify implementation of the assay)
- Choose appropriate controls
- Perform initial optimization studies to establish assay protocol and parameters before starting the validation

CLINICAL VALIDATION

Clinical validation assesses the clinical validity and clinical utility of the test in light of clinical characteristics of the disease/marker being tested. Data for clinical validation can be obtained from studies performed by the laboratory, or from studies reported in peer-reviewed literature or other reliable sources. CLIA requires laboratories to have a qualified laboratory director who is responsible for ensuring the clinical utility of the tests performed in his or her laboratory.

Elements of Clinical Validation

- Clinical Validity - the ability of a test to detect or predict the associated disorder (phenotype)
- Clinical Utility - the usefulness of the test in the diagnosis or treatment of patients
 - Must define testing purpose (screening, diagnostic, predictive, etc.)
 - Documented via literature review and/or independent establishment by the laboratory
- Clinical Sensitivity - the proportion of patients with the mutation/disease who have a positive test result.
 - Clinical sensitivity = True positive results ÷ (True positive + False negative)
- Clinical Specificity - the proportion of patients who lack the mutation/disease who have a negative test result.
 - Clinical specificity = True negative results ÷ (False positive + True negative)
 - *N.B.:* Predictive values are a function of the prevalence of the disease in the population being tested as well as the properties of the test [e.g., the higher the prevalence, the higher the likelihood that a positive result is a true positive]

TEST OUTCOME	CLINICAL CONDITION		
	POSITIVE	NEGATIVE	
POSITIVE	True Positive	False Positive	Positive Predictive Value TP / (TP + FP)
NEGATIVE	False Negative	True Negative	Negative Predictive Value TN / (TN + FN)
	Sensitivity TP / (TP + FN)	Specificity TN / (FP + TN)	

ANALYTICAL VALIDATION

NOTE: Please note that the following are only suggestions, not directives. The extent and type of validation that is needed for a particular test is left to the discretion of the laboratory director.

Accuracy (Trueness)

Definitions:

- Standards for the Reporting of Diagnostic Accuracy (STARD) definition: The amount of agreement between the information from the test under evaluation (the index test) and the reference standard (the best available method for establishing the presence or absence of the condition of interest)¹⁶

- The closeness of the agreement between the results of a single measurement and the true value of the analyte^{17, 18}

Precision

Definitions:

- The closeness of agreement between independent test/measurement results obtained under stipulated conditions^{17, 18}
- Getting the same results with repetition of the assay.

Elements of precision:

- Repeatability – this is referred to as intra-assay variation or within-run precision. It is the closeness of agreement between repeated tests of the same sample under the same operating conditions.
- Reproducibility – This is referred to as inter-assay variation or between-run precision. It is the closeness of agreement between the results of measurements when operating conditions are varied. All potential sources of variability should be considered. Typically, the same material is analyzed by the same protocol on different days, by different operators and with different equipment.
- Sources of variability that may have an impact on precision
 - Operator (most common source of variability)
 - Reagent lot (second most common source of variability)
 - Instrument
 - Sample concentration
 - Sample source
 - Run
 - Time of the day
 - Laboratory environment

Reportable Range

Definition: The span of test result values over which the laboratory can establish or verify the accuracy of the instrument or test system measurement response¹⁹.

Linear Range

Definition: The range where the test values are proportional to the concentration of the analyte in the sample⁶.

Reference Intervals

Definition: The range of test values expected for a designated population of individuals, e.g., 95 percent of individuals that are presumed to be healthy (or normal)²⁰.

Analytical Sensitivity

Definitions:

- The ability of a test to detect a mutation when that mutation is present.
 - $\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}}$
- Also used to refer to the lower limit of detection (LLOD) for the analyte of interest (i.e., the lowest concentration of analyte that the assay can consistently detect with acceptable precision),
- Or the lowest amount of starting material (nucleic acid) for an assay that will provide consistent results with acceptable precision.

Analytical Specificity

Definitions:

- The ability of a test to give a normal (negative) result in specimens that do not have the mutation being tested.
- $\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}}$

- Also used to refer to the ability of a test to detect only the intended analyte without cross-reacting with closely related analytes or potentially interfering substances. For example, a test that is intended to be specific for cytomegalovirus should not cross-react with herpes simplex virus 1.
- Sources of contamination, admixture, and substances that might interfere with analytical specificity:
 - Poor sampling
 - Maternal cell contamination in prenatal specimens
 - Cross contamination during sample processing
 - Inclusion of normal, non-diseased tissue with the diseased tissue of interest
 - Tissue from source addition to the desired sample
 - Bacteria
 - Endogenous substances (e.g. hemoglobin, cholesterol, triglycerides)
 - Exogenous substances (e.g. medications, anticoagulants, residual sample processing, stabilization reagents)

Potential interfering substances and cross-reacting nucleic acids that may have an effect on the accurate measurement/determination of the nucleic acid sequence of interest should be ruled-out through interference studies during validation.

REFERENCE MATERIALS AND CONTROLS

Reference materials (RM) are substances whose properties are sufficiently homogeneous and well established to be used for the calibration of the measuring system, the assessment of a measurement method, for assigning values to materials, or for quality control.

RM should be selected based on the needs of the assay, the methodology, and the availability of materials. Potential benefits and drawbacks should be evaluated with respect to each assay.

- Genomic DNA – mimics patient sample in term of complexity but can only control for one or a few alleles/genotypes or targets at a time
- Recombinant plasmids or synthesized oligonucleotides – do not resemble complexity of genomic DNA but can control for multiple alleles in a single reaction

Examples:

Human DNA, bacterial and viral genomic DNA, mitochondrial DNA, synthetic DNA, plasmids containing human DNA, amplicons, *in vitro* transcripts, synthetic oligonucleotides, recombinant DNA, phage and phage protein packaged nucleic acid, genetically modified cell lines

Resources: AMP maintains a collection of links to laboratory testing reference and validation materials and providers at http://www.amp.org/committees/clinical_practice/ValidationResources.cfm.

SUPPLEMENTAL GUIDANCE FOR ANALYTIC VALIDATION

NOTE: Please note that the following are only suggestions, not directives. The extent and type of validation that is needed for a particular test is left to the discretion of the laboratory director.

Accuracy (Trueness)

- Analyze known samples (either of known concentration or result, or both) and compare the result with that obtained by a reference (“gold-standard”) or comparative method, when the former is not available.
- Test samples that span the entire reportable range for quantitative assays and different possible genotypes for genotyping assays.
- For sequencing assays
 - It is important to establish that the test is capable of detecting appropriate representative types of DNA changes (e.g. point mutations, deletions, insertions).

- If the disease is uncommon, it may be necessary to obtain reference materials from other laboratories and/or commercial sources.
- However, the number of possible mutations essentially precludes the use of reference materials that cover every possible mutation.
- For quantitative assays, compare results between the new method and “reference” method or method already established in the lab. Evaluation of bias between new and comparative method) can be done in one of the following ways:
 - Calculation of average bias and comparison to CAP limits for passing proficiency testing.
 - Calculation of statistically significant difference in the mean by applying the t-test.
 - Use linear regression analysis:
 - Plot data (reference samples/method: x-axis; measured values/new method: y-axis)
 - Calculate linear regression statistics (ideally: slope=1, intercept=0 and r=0.99)
 - Establish criteria for accepting results (e.g. >95% confidence interval, +/- 2 SD)
- The appropriate number of specimens depends on many factors including, but not limited to:
 - complexity of the assay
 - frequency of targets/alleles in the intended use population
 - established accuracy of reference methods
 - whether test is FDA-approved, FDA-cleared, modified FDA-approved or LDP

Precision

- Within run: Run the same sample several times in one run
- Between runs: Run a set of samples in several different runs over several days
- Determine the mean, standard deviation and coefficient of variation (standard deviation ÷ mean)
- Precision studies should be carried out for the entire assay, as applicable, from extraction and amplification to detection.

- For Genotyping Assays:
 - For assays that utilize melting temperature (T_m): select control samples that cover the different genotypes (e.g. to identify prothrombin 20210G>A mutation, run at least one homozygous mutant, one heterozygous and one homozygous wild type sample, respectively). Run replicates on each run over. Calculate the average T_m and SD of the T_m. The T_m values have to be within the range of the manufacturer’s claim.
 - For assays that require peak size to determine number of repeats: e.g., for Fragile X by PCR, take 3 control samples of known size within the normal range (≤ 49 repeats), 3 “low” pre-mutations (50-79 repeats), and 3 “high” pre-mutations (80-200 repeats). Calculate the average size and SD. Acceptable range for Fragile X PCR +/- 3 repeats²¹.
 - Include representation of rare alleles to ensure that their presence can be detected with precision.
 - Test the ability of the system/assay to discriminate similar and adjacent mutations, where possible e.g.: *BRAF* V600E (c.1799T>A) and V600K(c.1798_1799GT>AA)
- For Quantitative Assays:
 - Take at least 3 sample concentrations (clinical samples or control samples) that cover the clinically important decision levels (e.g. for HCV quantitative assays a “low” control of ~100 IU/mL, a “medium” of ~5000 IU/mL, and a “high” of ~100,000 IU/mL). Depending on the assay the low concentration replicates can be two to four times the established Level of Detection (LoD), high concentrations should be close to the 99th percentile of tested concentrations/titers.
 - Example: A single operator testing five replicates at three concentrations (low, medium, high) run for 3 days using a single lot of reagent measured on the same instrument (total of 15 replicates per concentration will be generated) (CLSI, EP15-A2). It may be necessary to conduct multiple repeatability validation studies to cover all testing variables (see above).

- Use a spreadsheet to calculate mean value, standard deviation and %CV for within-run and between-run precision, and percent of agreement between tests performed under two different conditions (confidence interval should be calculated for the observed percent agreement). For FDA approved assays, compare to the manufacturer's claim; if higher %CV were obtained, then evaluate the cause.
 - Compare precision to clinically acceptable variation (e.g. for HCV quantitative assays, changes beyond 0.5 log are considered true changes and not intrinsic test variation).

Reportable Range

Qualitative Assays: For real-time PCR assays, a cutoff CT value can be determined using low positive sensitivity controls as well as negative validation samples. For fragment analysis assays (e.g. *NPM1*), positive controls can be tested to determine the mutant peak size range (e.g. 169-171bp). A positive result can be defined as any detectable and reproducible mutation that results in an amplification product that falls in the mutant peak size range. For Sanger sequencing and pyrosequencing assays, the reportable range is any nucleotide mutation identified in the codon(s)/exon(s) of the gene of interest that is being tested. This can be assessed by testing positive controls and/or positive inter-laboratory validation samples.

Quantitative Assays:

- Assay a series of standards or serial dilutions of a known standard or sample
- Graph the results
 - x-axis: Known values; y-axis: Measured values
 - Determine slope (ideal = 1.0) and intercept (ideal = 0), calculate regression

CLIA-88 requires that the reportable range does not exceed the range of the available calibrators

Reference Interval (Normal Values)

Normal range: Perform assay on normal controls (i.e., patients without the condition being tested). The number of samples to be tested is at the discretion of the laboratory director and will be dependent in part on the condition being tested and on the availability of appropriate control specimens.

- If clinically indicated for the condition being tested, include in the normal control range both males and females with a representative ethnic background and age distribution to the population being tested, pregnancy status, etc.
- May also consider including individuals with other medical conditions, if such conditions represent the clinical status of the patients who will be tested.

Abnormal range: Include in the abnormal control group individuals who are heterozygous and individuals who are homozygous, if possible.

Analytical Sensitivity

- Can be obtained by measuring a set of samples at different concentrations a given number of times. Excessive amount of analyte (e.g. nucleic acid) can cause incorrect genotype calls; insufficient amount of analyte can cause allele drop-out and false-negative results. False-negative rate can be determined by testing individuals who are known to have the condition being tested.
- Limit of Detection (LoD) - the lowest amount of analyte in a sample that can be detected, but not quantified as an exact value (WHO). It is a concentration of analyte above the limit of the blank that will be detected 95% of the time with a 95% confidence interval. Can be determined by testing serial dilutions of multiple replicates with a known concentration of the target substance in the analytical range of the expected detection limit.
- For multiplex assays LoD must be established for each target singly, as well as verified with other targets that could potentially be present in the same sample. If one target of multiplex assay is present at a high level, the detection of another target present at low levels could be potentially impaired (e.g. fetal DNA in maternal blood, maternal cell contamination in fetal specimens, mosaics or minimal residual disease).

- Compare results with those of a “gold-standard” reference method or of another validated method in use in the lab.

Analytical Specificity

- Testing of potential interference should be conducted at the highest concentration that would occur in a clinical setting. The maximum amount of a potentially interfering substance the assay can tolerate without causing actual interference or adversely affecting the test results should be determined. Interference studies can be conducted by spiking the specimens with interfering agents (spiked vs. unspiked). Studies should be performed for each specimen matrix used in the assay.
 - RNA copurified with varying levels of DNA and vice versa.
 - Residual reagents such as organic solvents, wash buffers, residual high salt concentrations, heparin, and others.
 - Cross reactivity –To determine if analytes other than the ones the assay is designed to measure cause false positive results, a panel of closely related organisms/alleles should be assessed.
- False-positive rate can be determined by testing individuals known to have condition different than that being tested, but that is similar enough to be in the differential diagnosis (e.g.: a test that is intended to be specific for cytomegalovirus should not cross-react with herpes simplex virus 1)..
- Compare results with those of a “gold-standard” reference method or of another validated method in use in the lab.

Documentation

All steps of the Analytical Validation should be carefully documented.

ONGOING QUALITY CONTROL MEASURES

- Controls should be included (both positive and negative controls and a blank, lacking nucleic acid template), as appropriate for each assay. Quantitative assays should include negative, low-positive, high-positive controls and a blank.
 - The positive control should have a clinically relevant amount of analyte.
 - The negative control should contain other nucleic acid targets that would be expected to be present in the patient sample.
 - Controls should be present in a biologically matrix that simulate clinical samples.
 - Controls should be processed in the same manner as patient samples.
 - It is important to consider controlling each step from primary sample to result.
 - Failure of any of the controls invalidates the run and repeating the entire run should be considered in this case.
 - The blank should contain the complete reaction mixture EXCEPT nucleic acids or for assays that start with RNA that is processed to cDNA, use RNA that is processed without reverse transcriptase added as the blank.
 - Amplification of an internal nucleic acid target (either endogenous or spiked) is recommended to ensure that inhibitors are not interfering with the assay.
- Standards (e.g., size markers) should be used in each run that involves size separation.
- Proper calibration of the instruments used in the assay should be maintained.
- Routine preventive maintenance of all instruments (e.g., thermocyclers, pipettes, etc.).
- Testing of new lots of reagents - New lots of all reagents should be tested side-by-side with the old reagent, prior to implementation.
- Proficiency Testing
 - Labs should participate in national testing programs (e.g., CAP) for all tests they perform.
 - If no proficiency test is offered, labs should participate in an alternative assessment at least twice per year (per CLSI). An inter-laboratory exchange is preferable. CAP has a sample exchange registry for assays not offered in its commercially available proficiency testing.
- Assessment of continued technologist competency.

- Documentation - the laboratory must keep results of validation studies as long as the protocol is in use, and for at least two years after the protocol is retired.
- Reporting/Laboratory Information System
 - The test report should reflect the reason for the patient's referral.
 - If pre-written interpretations ("canned comments") are used, the laboratory director should ensure that the correct test interpretation is issued.

ONGOING QUALITY ASSURANCE MEASURES

- Regular review of normal and abnormal results (e.g., review of percentage of positive reactions, by virus or other analyte)
- Review of turnaround times
- Review of rejected specimens
- Annual Quality Improvement project(s)

DISCLAIMER

The AMP Clinical Practice Guidelines and Reports are developed to be of assistance to laboratory and other health care professionals by providing guidance and recommendations for particular areas of practice. The Guidelines or Report should not be considered inclusive of all proper approaches or methods, or exclusive of others. The Guidelines or Report cannot guarantee any specific outcome, nor do they establish a standard of care. The Guidelines or Report are not intended to dictate the treatment of a particular patient. Treatment decisions must be made based on the independent judgment of health care providers and each patient's individual circumstances.

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VALIDATION SUMMARY

ASSAY _____

NEW / MODIFIED

FDA-APPROVED or CLEARED / LDP

CHECKLIST FOR DOCUMENTATION OF VALIDATION STUDIES

Assay Design

- Define the requirements of the test
 - e.g., intended use, target population, test method and expected performance characteristics

Clinical Validation

- Documentation of the clinical validity and clinical utility of the test in light of clinical characteristics of the disease/marker being tested
 - e.g., prevalence of the disease in a relevant population, prevalence of sequence or copy number variants
- Clinical sensitivity (the proportion of patients with the disorder that will be positive by this test)
- Clinical specificity (the proportion of patients without the disorder that will be negative by this test)

Analytical Validation

- Optimization of assay on an adequate number of each of the expected specimen types
- Accuracy (getting the right results)
 - e.g., Parallel testing with an alternate method
- Precision (the ability of a test to produce the same result on repeated testing of the same target)
 - e.g., Replicate testing within and between runs, as well as with different technologists if possible
- Reportable Range (the span of results that the lab can verify)
- Reference Interval (result range in normal individuals)
- Analytical sensitivity (the lowest amount of target nucleic acid that can be detected by the assay)
 - e.g., Testing various dilutions of a known target in a relevant matrix
- Analytical specificity (the ability of a test to give a negative result when the target nucleic acid is not present)
 - e.g., Testing of an adequate number of negative specimens
- Reporting criteria established
- Standard operating procedure written
- Final report template prepared
- Ongoing QC and proficiency testing procedures established and documented

APPROVED: Laboratory Director _____ Date: _____