

VERIFICATION AND VALIDATION TOOLKIT

Determining Performance Characteristics of Quantitative Assays

To ensure correct diagnosis and treatment, clinical laboratory testing must be accurate and reliable. A key component of the quality assurance process is the verification or validation of new instruments and tests to confirm their ability to perform prior to implementation.

The Verification and Validation Toolkit walks users through this process and provides additional resources, templates and examples for use in the laboratory.

Find the complete toolkit at aphl.org/VV-Toolkit

The toolkit has eight sections:

1. Verification and Validation 101
2. Verification and Validation Process Checklist
3. Obtaining Appropriate Test Samples
4. Qualitative Assays
5. **Quantitative Assays**
6. Related Processes
7. Safety Considerations and Risk Assessments
8. Cost Analysis and Budget

Quantitative assays are methods that provide a numerical value to the submitter and therefore have additional requirements. Those additional requirements include additional verification or validation data, calibration (may use a standard curve), and the use of quantitative controls.

For quantitative tests, the manufacturer’s limits of detection, linearity, reportable range and precision must all be validated or verified by the lab. **Table 1** shows the updated requirements for quantitative tests.

Table 1. Summary of Performance Characteristics Required Depending on Qualitative Test Type¹

Test Type	Accuracy	Precision	Analytical Sensitivity	Analytical Specificity	Reportable Range	Reference Range
FDA Approved	Required	Required			Required	Required
FDA Cleared	Required	Required			Required	Required
FDA Modified or LDT	Required	Required	Required	Required	Required	Required
FDA Authorized (EUA)*	Required	Required			Required	Required

* Requirements may vary depending on EUA. Performance characteristics will be defined by the EUA instructions for use and the laboratory director. Instructions should include requirements for verification or validation.

Calibration and Statistical Calculations

Calibration is establishing, under specified conditions, the relationship between reagent system or instrument response and the corresponding concentration of an analyte. If the quantitative test is FDA-approved or cleared, calibration must be carried out according to the manufacturer’s instructions. If calibrating an FDA-modified or laboratory-developed test (LDT), ensure that high quality, matrix appropriate materials are used that will provide ideal target values. Materials may need to be purchased from qualified vendors.

Through the verification process, the laboratory defines the frequency for calibration performance as well as the type, number, and concentration of calibration materials used to monitor, detect error, and evaluate method performance. The frequency for calibration performance must not be less than the frequency specified in the manufacturer’s instructions.

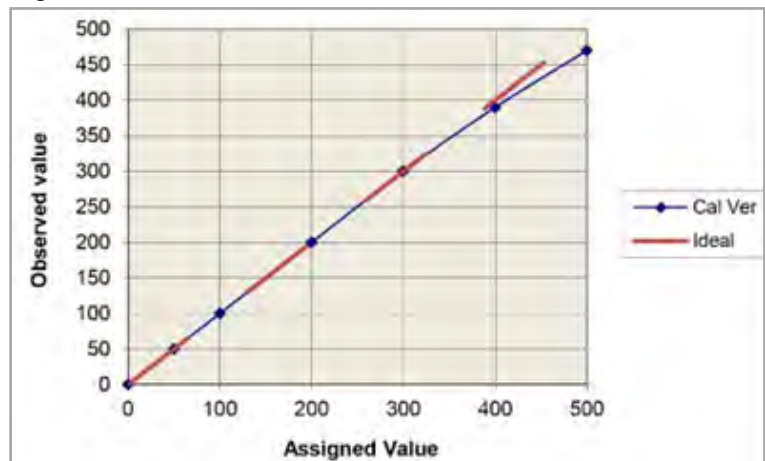
Calibration requirements vary by assay and depend on the level of FDA approval, but a minimum is typically duplicate calibration runs. FDA approved or cleared tests may allow for a single run. Lower concentrations of calibrators may require more replicates due to the potential for greater variability. Use calibration data to perform regression analysis. Examples are listed below, but the requirements for each method may vary.

Graphical Data Assessment

Westgard QC describes a graphical data assessment, seen in **Figure 1**.² Plot the measurement results on the y-axis vs the assigned values on the x-axis. Draw a 45° line of identity, then draw a “point-to-point” line for the measurement results. Compare the two lines.

- **The coefficient of determination (R^2)** is a measure of how well the regression model fits the observed data. R^2 should be close to 1 (i.e., 0.991 is a close fit).
- **The coefficient of variation (CV)** can be used to express the precision and repeatability of an assay. The ideal CV is <10-15% and generally should not exceed 20%.
- Calculating the slope is another measure of comparability. The slope should be close to 1.

Figure 1. Calibration Verification



Coefficient of Variation

CV Calculation:³ The CV is the ratio of the standard deviation to the mean. CV is expressed as a percentage. The ideal CV is <15%, and generally should not exceed 20%. Use the formula in **Figure 2** to calculate the CV.

Figure 2. Calculating the Coefficient of Variation

$$CV \% = 100 \times \frac{\text{Standard Deviation}}{\text{Mean}}$$

Method Validation Data Analysis Tool Kit

Westgard QC offers a Method Validation Data Analysis Tool Kit and an online Paired-Data Calculator.⁴ This calculator can be used with data from a comparison of methods experiment to calculate linear regression statistics (slope, y-intercept, and standard deviation about the regression line, $s_{y/x}$), and the correlation coefficient (r , Pearson product moment correlation coefficient); t-test statistics (average difference between two methods or bias_{meas}; SD_{diff} , standard deviation of the differences between the two methods). It can also be used to provide a “comparison plot” that shows the test method results on the y-axis versus the comparative method results on the x-axis, as well as a “difference plot” that displays the difference between the test minus comparative results on the y-axis versus the comparative method result on the x-axis.

Quantitative Controls

Quantitative controls are required for verification or validation and for testing runs following approval of the method. The minimum recommendation is a low positive, high positive, and a negative control. Depending on the assay, the laboratory could customize its QC plan using an Individualized Quality Control Plan (IQCP).^{5,6} If controls fail to produce the expected results, the run must be rejected, and the failure should be investigated to identify the cause.

Accuracy

This guidance follows the accuracy requirements for qualitative assays; however, the accuracy study must consider the quantitative results and span the analytical measurement range (AMR). Appropriate samples include commercially available calibrators/calibration or quality control materials with known values, proficiency testing materials that have established values, or previously tested patient specimens with established values. Contrived samples may be used if the appropriate sample type or quantity is not available. Sample mixtures may also be used to achieve the appropriate quantitative value for testing.

Precision

Precision for quantitative assays includes the same structure (inter- and intra- assay measurements) and calculations (%CV) as qualitative tests. Runs should include samples with at least two levels of analyte concentrations. Prior to starting, criteria for identifying and handling outliers should be established to ensure any operational problems do not distort the data. If the test does not routinely include runs, four samples should be run as two sets of pairs at different times on the same day. Those results should be treated as if they were two results resulting from the same run. If the test includes a run that can be completed multiple times in a single day, two runs (separated by at least two hours) with two samples (run in duplicate) should be analyzed with at least one quality control sample and 10 patient samples (if possible) each day. These should be repeated for 20 days with the order of the test materials and quality control samples changed each run or day.⁷

Analytical Sensitivity

Analytical sensitivity establishes the limit of detection for a quantitative assay. Dilutions of a known concentration should be tested until the method or instrument no longer detects the presence of the analyte within a matrix. If the laboratory is evaluating a modified test system, it is acceptable to use the manufacturer's stated lower limit if it can be shown that the modification had no effect on the lower limit threshold. Once the LoD for an LDT is established or when verifying a manufacturer's limit of detection claim, it is recommended to test replicates of a number of samples with concentrations both below and above the limit of detection. [CLSI EP17-A2](#)⁸ recommends testing four low positives and four blank samples. The samples should be run in duplicate or triplicate over three days with a recommended minimum of 20 measurements for each sample concentration to verify a manufacturer claim, and 60 measurements to establish the LoD. However, the exact number of samples to use should be determined on a case-by-case basis with input from the QAO, supervisors, or the laboratory director. The LoD should be defined as the concentration in which the assay can distinguish positive from negative samples 95% of the time.

Analytical Specificity

Analytical specificity is the evaluation of cross-reactivity by testing a panel of similar, potentially interfering organisms, substances, or analytes to assess constant systematic error. The test agents or substances should include as many organisms or analytes as possible that may be found in the relevant test sample or that cause the same symptoms as the target agent. Consider potential sources of variability that could affect the assay (i.e., matrix composition, lot-to-lot variability, temperature, etc.) and include them in the design of the verification or validation. The recommended number of samples to use are three to five containing each of the potentially interfering or cross-reactive test organisms, analytes or substances to test. Results should correlate with an expected value $\geq 95\%$. If cross-reactivity is observed, assay conditions may need to be adjusted or reevaluated. In instances where cross-reactivity cannot be eliminated, it must be noted as a limitation of the assay. An inhibition control may need to be included in assay runs where inhibition is prone and needs to be monitored (i.e., molecular assays direct from specimens).

Reportable Range

Reportable range refers to the range of values that can be accurately measured by the test. Calibrators may be used to establish reportable range. For quantitative tests, the manufacturer's reportable range must be verified by the testing laboratory. No quantitative value can be reported that falls outside of the validated range. Other considerations for quantitative assays are to consider how to report numerical results in order to provide the most clinically meaningful information (i.e., log scale vs. integers; 5.30–5 vs 200,000–100,000). Also, some assays may include a cutoff or threshold value that correlates to disease while many do not.

Reference Range

Reference intervals or reference ranges are the span of results expected for a group of healthy individuals who are disease or analyte free. These are results that are considered “normal” for a healthy individual. Reference values for an infectious disease test may not be applicable. However, for values reported in quantitative assays, such as blood chemistry assays, a set of known healthy samples may need to be tested to establish the reference intervals. It is acceptable to use manufacturer or published reference intervals provided they are appropriate for the laboratory's patient population. If this is done, the verification/validation summary should document the source of the reference intervals and an explanation of their evaluation for appropriateness against the laboratory's patient population. If, after testing commences, it is determined that the established reference interval is no longer appropriate, it needs to be reevaluated, and documentation of the reevaluation retained with the original verification/validation records.

Ongoing Verification of Quantitative Assays

Calibration Verification

Calibration verification must be performed every six months (or more frequently if specified in the manufacturer's instructions)⁹ and:

- When changes are made to the assay, instrument or overall test system
- When there are major shifts in QC results
- When proficiency testing is inconsistent
- When quality assurance activities indicate discrepant results

There are exceptions to calibration verification requirements:

- Instruments that are factory or manufacturer calibrated do not require calibration verification.
- If the test system's calibration procedure includes three or more levels of calibration material, and includes a low, mid, and high value, and is performed at least once every six months, then the requirement for calibration verification is also met.

The laboratory defines acceptance or rejection criteria.

Analytical Measurement Range Verification^{10,11,12}

- Analytical measurement range (AMR) verification must be performed every six months and when changes are made to the assay.
- If calibration includes low, midpoint and high values spanning the AMR, additional testing may not be required.
- Control materials may be used.

Additional Quantitative Assay Resources

- [APHL CLIA-compliant Analytical Method Validation Plan and Template for LRN-C Laboratories](#)
- [APHL CRO Breakpoint Implementation Toolkit](#)
- [CLSI 2023 Breakpoint Implementation Toolkit](#)

References

- 1 Code of Federal Regulations. 42 CFR 493.1253 Standard: Establishment and verification of performance specifications. National Archives and Records Administration; 2003. Accessed Oct 1, 2023 from: www.ecfr.gov/current/title-42/chapter-IV/subchapter-G/part-493/subpart-K/subject-group-ECFRc96daeadd380f6ed/section-493.1253
- 2 Westgard QC. James O. Westgard. Calibration Verification: Defining Criteria for Acceptable Performance. May 2016. Accessed October 1, 2023 from: www.westgard.com/cal-verification-criteria.htm
- 3 Bio-Rad UnityWeb. Coefficient of Variation. Accessed October 15, 2023 from: unityweb.qcnet.com/Documentation/Help/UnityWeb/402.htm
- 4 Westgard QC. Method Validation Data Analysis Tool Kit. Accessed October 15, 2023 from: www.westgard.com/mvtools.htm
- 5 CMS CLIA. State Operations Manual Appendix C - Survey Procedures and Interpretive Guidelines for Laboratories and Laboratory Services, Rev. 166, 02-03-17. IQCP is on page 198. Accessed Jan 15, 2024 from: www.cms.gov/regulations-and-guidance/guidance/manuals/downloads/som107ap_c_labpdf.pdf
- 6 CDC Laboratory Quality Tools and Resources. Individualized Quality Control Plan (IQCP). Accessed Oct 15, 2023 from: www.cdc.gov/labquality/iqcp.html
- 7 CLSI. Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline—Third Edition. CLSI document EP05-A3. Wayne, PA: Clinical and Laboratory Standards Institute; 2014. Available from: clsi.org/standards/products/method-evaluation/documents/ep05/
- 8 CLSI. Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures; Approved Guideline—Second Edition. CLSI document EP17-A2. Wayne, PA: Clinical and Laboratory Standards Institute; 2012. Available from: clsi.org/standards/products/method-evaluation/documents/ep17/
- 9 CMS CLIA. Calibration and Calibration Verification Brochure. Accessed Jan 14, 2024 from: www.cms.gov/files/document/clia-brochure-calibration-and-calibration-verification-april-2006.pdf
- 10 CLSI. Evaluation of Qualitative, Binary Output Examination Performance, 3rd ed. CLSI guideline EP12. Clinical and Laboratory Standards Institute, 2023. Available from: clsi.org/standards/products/method-evaluation/documents/ep12/
- 11 Validation and Implementation of Quantitative Molecular Assays, Morgan A. Pence April 10, 2019, Molecular Diagnostics 2019, Sponsored by DiaSorin Molecular LLC. Accessed October 1, 2023 from: www.youtube.com/watch?v=k0N_toug60Q
- 12 CMS CLIA. Calibration and Calibration Verification Brochure #3. Accessed October 1, 2023 from: www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/downloads/6065bk.pdf



Association of Public Health Laboratories

The Association of Public Health Laboratories (APHL) works to strengthen laboratory systems serving the public's health in the US and globally. APHL's member laboratories protect the public's health by monitoring and detecting infectious and foodborne diseases, environmental contaminants, terrorist agents, genetic disorders in newborns and other diverse health threats.

7700 Wisconsin Avenue, Suite 1000 Bethesda, MD 20814 | 240.485.2745 | www.aphl.org

© Copyright 2024, Association of Public Health Laboratories. All Rights Reserved.

This publication was supported by Cooperative Agreements #NU600E000104, 100% funded by the US Centers for Disease Control and Prevention (CDC). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of CDC or the US Department of Health and Human Services.