Validation and QC Fundamentals for Qualitative and Quantitative Assays

Teri Oldaker CLS, SCYM\textsuperscript{CM} (ASCP)

ICCS Course 2021
Baltimore, MD
Objectives

• To define and understand validation fundamentals
• To clarify regulatory categories and impact on validation/verification
• To understand each performance specification required
• To understand the purpose of quality control and flow specific requirements.
Why validate?

• Provides confidence that an assay will yield reliable results.
• Confirm and document the method works as intended, in your lab
• Proves that with consistent input yield consistent outputs.
• Validation is required in regulatory environments (CLIA)
Validation Requirements
Qualification, Validation and Verification

- **Validation (analytical):** Demonstrate a method is suitable for intended use. *Establish* performance specifications for assay. (analytical)

  - **Qualification:** To determine *the components* used in a method are suitable for use.
    - Instrument
    - Reagents
    - Personnel

- **Verification:** Demonstrate that a *previously validated* method is suitable for use. *Verify* the established performance specifications

- **Validation (clinical):** Will test results benefit the patient?
Validation vs Verification

• FDA cleared (IVD)
• Laboratory Developed Test (LDT)

1) Commercially Distributed Test Pathway (IVD):
   “test kit” manufactured for distribution to multiple labs
   “test kit” distributed to patients, hospital, or clinical lab
   “enforcement discretion”
   LDTs (lab developed tests) enter the market without review

2) Lab Developed Test (LDT) Pathway:
   Test designed, manufactured, and used in a single lab
   “test kit” manufactured for distribution to multiple labs
   “enforcement discretion”
   LDTs (lab developed tests) enter the market without review

• Verification confirms already established performance specifications
• Validation establishes performance specifications
Data Type

- **Quantitative**: calibration curve or reference standard

- **Semi Quantitative (Quasi-quantitative)**: estimate, not assessed with calibration curve

- **Qualitative**: Detects presence or absence. Descriptive, not numeric
Prior to Validation/Verification

- Instrument (LDT)
  - Qualified (IQ/OQ/PQ)
  - Optimized

- Assay (LDT)
  - Developed
  - Optimized

- Documentation approved
  - Standard Operating Procedure
  - Validation Plan

---

Design  Measure  Evaluate  Validate  Control (QC)
Set conditions and procedures
Maintain established specifications over time
Great 8/10 color panel!
Say it, Do it, Prove it
Steps of Validation

- Define Intended Use
- Regulatory category
- Clarify Data Type
- Write and Approve Validation Plan
- Establish Standard Operating Procedures (SOP)
- Perform Validation Experiments per Plan
- Write and Approve Validation Report
- Train staff
- Launch assay
Say it, Do it and Prove it

• Validation Plan (Say It!)

• Validation Execution (Do It!)

• Validation Reports (Prove It!)
Validation Plan

• Materials and Methods
  • Description of the method including reportable results
    • Reagents
    • Equipment and Software
  • Specimens to be used for validation studies

• Experimental Approach
  • Description of experiments
  • Description of the statistical analysis
  • Acceptance Criteria (based on quality requirements)

Table 5: Abnormal Sample types for validation:

<table>
<thead>
<tr>
<th>Disease</th>
<th>Sample Type</th>
<th>Anticoagulant</th>
<th>Number of cases</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoma</td>
<td>Fresh tissue</td>
<td>RPMI</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>B-CLL/SCL</td>
<td>PB</td>
<td>EDTA and heparin</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>B-CLL/SCL</td>
<td>BM</td>
<td>Heparin</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>PB</td>
<td>EDTA and heparin</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>BM</td>
<td>Heparin</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>PB</td>
<td>EDTA and heparin</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>BM</td>
<td>Heparin</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Myeloma</td>
<td>BM</td>
<td>Heparin</td>
<td>2</td>
<td>As available</td>
</tr>
<tr>
<td>Senear</td>
<td>PB</td>
<td>EDTA and heparin</td>
<td>2</td>
<td>As available</td>
</tr>
<tr>
<td>Other</td>
<td>PB/EM tissue</td>
<td>as available</td>
<td>2</td>
<td>As available</td>
</tr>
</tbody>
</table>
Validation Report

- Summary of experiments
  - Tabular representation of data
  - Comparison of results vs acceptance criteria
- Raw data
  - In appendix or study binder
- Deviations/explanations
- Conclusions
  - Recommendations for use

### EXECUTIVE SUMMARY OF VALIDATION RESULTS

<table>
<thead>
<tr>
<th>Qualitative Results</th>
<th>Quasi-quantitative Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection of disease</td>
<td>% abnormal cells</td>
</tr>
<tr>
<td>Qualitative accuracy</td>
<td>100% concordance</td>
</tr>
<tr>
<td>Quasi-quantitative accuracy</td>
<td>R2 = 0.983</td>
</tr>
<tr>
<td>Diagnostic Specificity</td>
<td>100% concordance</td>
</tr>
<tr>
<td>Diagnostic Sensitivity</td>
<td>100% concordance</td>
</tr>
<tr>
<td>Intra assay Precision</td>
<td>100% concordance</td>
</tr>
<tr>
<td>Intermediate Precision</td>
<td>100% concordance</td>
</tr>
<tr>
<td>Inter instrument Verification</td>
<td>100% concordance</td>
</tr>
<tr>
<td>Sample Stability</td>
<td>in process</td>
</tr>
<tr>
<td>Ab Cocktail Stability</td>
<td>in process</td>
</tr>
</tbody>
</table>

### 10 color assay

<table>
<thead>
<tr>
<th>Normal</th>
<th>Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>0</td>
</tr>
</tbody>
</table>

### 5 color assay

<table>
<thead>
<tr>
<th>Normal</th>
<th>Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>19</td>
</tr>
</tbody>
</table>
Validation/Verification Parameters

<table>
<thead>
<tr>
<th>Verification (IVD)</th>
<th>Validation (LDT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>✓ Verify Accuracy</td>
<td>✓ Accuracy</td>
</tr>
<tr>
<td>✓ Precision/Reproducibility</td>
<td>✓ Precision/Reproducibility</td>
</tr>
<tr>
<td>✓ Verify manufacturer’s reference range*</td>
<td>✓ Detection capability (analytical sensitivity)</td>
</tr>
<tr>
<td>✓ Stability (if exceeds insert)</td>
<td>✓ Selectivity (analytical specificity)</td>
</tr>
<tr>
<td>✓ Must follow manufacturers SOP</td>
<td>✓ Sample and Reagent stability</td>
</tr>
<tr>
<td></td>
<td>✓ Establish reference range*</td>
</tr>
<tr>
<td></td>
<td>✓ Calibration and control procedures</td>
</tr>
</tbody>
</table>

CLIA ‘88 Subpart K: 493.1213
Accuracy (Agreement)

• **Accuracy**: the closeness of agreement between the average value obtained from a large series of test results when compared to an accepted reference standard.

• No true standards or reference material in flow, accuracy cannot be assessed

• Can measure agreement
  • Inter-laboratory comparison
  • Verification from confirmed diagnosis by alternative methods
  • Comparison against predicate methodology
  • Testing reference standards or reference materials**
Agreement (Quantitative Assays)

- Sample size covers broad range of values
- Use multiple runs
- Ideally, run at same time

Statistical analysis
- $R^2$  Correlation of results
- Bland-Altman Analysis (bias from mean)
Correlation of Results

• Commonly used in chemistry assays
• Used to compare values of two methods
• $R^2$ - correlation coefficient
  • Strength of the relationship
  • Perfect correlation, $R^2 = 1.00$
  • Ideal criteria $R^2 \geq 0.95$
• Y intercept – bias
• Slope – proportional error

$R^2$ - May not always indicate good agreement
Bland-Altman Analysis (difference plot)

• Better for measuring agreement
• To determine if **bias** between two methods
• Compares difference between 2 measures to the mean
• Negative or positive bias visible
• Easier to see bias than with correlation
• Acceptability = 95% limit of agreement
Agreement (Qualitative Assays)

• Known positive and negative cases are compared by two methods. Concordance and disagreement are determined.

<table>
<thead>
<tr>
<th>Candidate test results</th>
<th>Comparative test results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>a</td>
</tr>
<tr>
<td>Negative</td>
<td>c</td>
</tr>
<tr>
<td>Total</td>
<td>a + c</td>
</tr>
</tbody>
</table>

• Concordance = (a+d)/(a+b+c+d) x 100%
• Selectivity or Negative Percent Agreement (NPA) = d /(d+b) X 100%
• Detection capability or Positive Percent Agreement (PPA) = a /(a+c) X 100%

• a = positive results concordance   b = negative result disagreement
• c = positive result disagreement   d = negative result concordance
Agreement (Qualitative Assays)

• Display data on a 2 X 2 contingency table
  • Concordance
    • Concordant values = 37
    • Total N = 40
    • % concordance = 37/40 = 93%

• Selectivity/NPA
  • (18/19) X 100% = 95%

• Detection capability/PPA
  • (19/21) x 100% = 90%
Precision/Reproducibility

• Precision is the dispersion of replicate measurements using “conditions of measurement”.
  • Intra assay - no variables (repeatability)
  • Inter assay - between runs (intermediate precision)
  • Inter operator - between operators (reproducibility)
  • Inter instrument - between instruments (reproducibility)
• Quantitative measured by the % coefficient of variation (CV)
• Qualitative measured by concordance of results
Experimental Design - Precision

- Challenges in evaluating precision
  - Limited sample stability
  - Limited sample volume and availability
  - Chemistry: run same 20 samples for 20 days

- Factorial Design Strategy
  - Multiple factors are evaluated in a single experiment
  - Rather than each factor evaluated independently
  - Evaluate different runs on same day
Detection Capability (analytical sensitivity)

- A set of performance attributes that characterize measurement accuracy in the low-end region of the measurement interval.

- How well the assay performs with known positives.
  - **Limit of Blank (LOB):** Highest signal in the absence of the measurand
  - **Limit of Detection (LOD):** The ability to detect measurand where 95% of the low level is above the LOB.
  - **Lower Limit of Quantitation (LLOQ):** The lowest concentration that can be quantitated with acceptable precision.
Detection Capability (analytical sensitivity)

• **LOB**
  • 10 normal donors
  • LOB = Mean of blank + 1.645 SD

• **LOD** (Estimate and/or Verify)
  • LOD = Mean of Blank + 3 SD
  or
  • 5 normal donors and 5 low positive samples,
  • Verification of results
  • <5% blank exceed LOB; <5% low + fall below LOD

• **LLOQ**
  • Serial dilutions run in triplicate.
  • Verify recovery.
  • Verify precision (<10%, or <30% near LLOQ)
Selectivity (analytical specificity)

- Ability to measure the intended measurand while avoiding cross-reactivity of interference.
- How the assay runs with known negatives
- Generally accomplished during panel design
- What impacts selectivity:
  - selection of cellular antigens
  - evaluation of Ab clones, reagent titration
  - evaluation of wash steps, buffers
  - gating strategy to identify subpopulations

Selectivity (analytical specificity)

- At validation, verify each antibody marks as expected
- Use TDS for expected reactions
  - Qualitative
    - Positive and negative on selected cell populations. 100% concordance is expected
    - Use NPA calculation (agreement)
  - Quantitative
    - Select samples across reportable range

### New Antibody Verification of Specificity

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Expected Positive expression*</th>
<th>Actual results</th>
<th>Pass/Fail</th>
<th>Expected Negative expression*</th>
<th>Actual results</th>
<th>Pass/Fail</th>
<th>Tech</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B lymphocytes</td>
<td>Positive</td>
<td>Pass</td>
<td>Neutrophils</td>
<td>Negative</td>
<td>Pass</td>
<td>DC</td>
</tr>
<tr>
<td>2</td>
<td>B lymphocytes</td>
<td>Positive</td>
<td>Pass</td>
<td>T lymphocytes</td>
<td>Negative</td>
<td>Pass</td>
<td>DC</td>
</tr>
<tr>
<td>3</td>
<td>B lymphocytes</td>
<td>Positive</td>
<td>Pass</td>
<td>Monocytes</td>
<td>Negative</td>
<td>Pass</td>
<td>DC</td>
</tr>
</tbody>
</table>

* Expected expression obtained from Vendor Technical Data Sheet

Comments: CD19-FITC marks as expected

Evaluation Date: __6/25/2019____________  Technologist: DC
Pathologist Review: __NZ M.D.______________________________
Stability

• Lack of variability in the measured analyte relative to time, temperature and anticoagulant

• Sample stability
  • Time of draw to assay
  • Time between stain and acquisition

• Reagent stability
  • Cocktail stability

• Verify equivalent results can be obtained when compared to reference*
  • Freshly drawn samples*
  • Freshly prepared reagents*
Experimental design - Stability

- ≥3 samples (diseased and normal)
  - Sample: run same sample at different time points
  - Cocktail: run same sample using fresh cocktail vs aged cocktail
- Compare results with baseline timepoint
- Qualitative
  - Concordance across time points.
  - Cocktail compare MFI for change ≤ 10% change
- Quantitative
  - Change ≤ inter assay precision
  - MFI of each population ≤ 10% change

CLSI EP25
Reference Intervals

• The range of values obtained on specimens from normal donors assayed under conditions used for patient samples.

• Qualitative assays: NA

• Quantitative assays:
  • Establish full reference interval $N=120^*$
  • Verify a published reference range $N = 20$

CLSI document EP28*
## Laboratory Initiated Assay Revisions

<table>
<thead>
<tr>
<th>Extent of change</th>
<th>Example of change</th>
<th>Action required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minor operational</td>
<td>Change in simple reagent (buffer)</td>
<td>Reagent qualification n=5</td>
</tr>
<tr>
<td>Moderate operational</td>
<td>Change antibody same fluorochrome FMC7 to CD200</td>
<td>Verify signal strength n=10</td>
</tr>
<tr>
<td>Moderate operational/clinical</td>
<td>Change in antibody different fluorochrome – no compensation issues</td>
<td>Verify equivalent selectivity and detection capability n=20 (5 normal, 15 abnormal)</td>
</tr>
<tr>
<td>Moderate operational/clinical</td>
<td>Change in antibody different fluorochrome – compensation issues</td>
<td>Full LDT validation. If quantitative include precision LOB, LOD and LLOQ</td>
</tr>
<tr>
<td>Moderate operational/clinical</td>
<td>Add 1 or more novel antibodies, adding additional colors (8 color to 10 color) Modifying an IVD assay procedure</td>
<td>Full LDT validation . If quantitative include precision LOB, LOD and LLOQ</td>
</tr>
</tbody>
</table>
Quality Control (QC)

A set of procedures performed by the laboratory staff for the continuous and immediate monitoring of laboratory work in order to decide whether the results are reliable enough to be released.

Assay specific QC should be designed to verify critical components of the assays intended use.

*Validation:* Are you running the right assay?

*Quality Control:* Are you running the assay right?
Flow Cytometry Quality Control

- Instrument
- Reagents (Antibody and other reagents)
- Procedure/Method
- Specimen integrity
- Patient internal controls
Assay specific QC

• Instrument
  • Verify assay specific voltages and compensation

• Assay
  • Quantitative assays: precision criteria is aligned to assay inter assay precision
  • PNH: verify periodically your LOB by running normal sample
  • Verify normal ranges are consistent over time
  • Ploidy and SPF verify linearity

• Internal
  • Known normal populations should mark as expected
Documentation Hierarchy

A. Policies “what to do”
B. Processes “how it happens”
C. Procedures “how to do it”
D. Quality Records “evidence it was done”

“If you didn’t document it, it didn’t happen”
Conclusion

- Cell based assay validations require a different approach
- Regulatory category defines the validation experimental design
- Data type defines the experimental design and statistics
- Statistical evaluation and documentation are essential.
- Quality control is used to ensure the performance does not change over time.
Additional Resources

• Validation of Cell-Based Fluorescence Assays: Practice Guidelines from the International Council for Standardization of Haematology and International Clinical Cytometry Society. Cytometry Part B: Clinical Cytometry Special Issue volume 84B:2013


  - ISAC/CYTOU Webinar: Validation the Key to Translatable Flow Cytometry a Three-part Webinar Series 2018
    - Instrument Qualification
    - Method Validation - Overview, Concepts
    - Method Validation - Planning and Execution
Additional Resources

Cytometry.org (ICCS website)

Quality and Standards Modules [https://www.cytometry.org/web/quality.php#acordion_13]
- Module 10: Verification of PNH assay sensitivity
- Module 16: Antibody cocktail validation

Educational presentations and videos


Newsletter articles
Questions?