Do Your Flow Cytometric LDTs Conform to the ICSH-ICCS Validation Guidelines?

Fiona E. Craig, MD
University of Pittsburgh School of Medicine
How should LDTs be validated?

• Accuracy
• Specificity
• Sensitivity
• Precision
• Linearity
• Carryover
• Reportable range
• Reference range
• Stability
How should LDTs be validated?

Hemoglobin Standard

This is the second and final report on a proposal to establish a certified standard for general use.

Division of Medical Sciences, National Academy of Sciences-National Research Council

In 1953 the Hemoglobin Study Section of the National Institute of Health requested the Division of Medical Sciences of the National Academy of Sciences-National Research Council to explore the possibilities of establishing a hemoglobin standard for general use throughout the country. In response to this request, the Division, in 1954, organized an ad hoc panel for the establishment of a hemoglobin standard under its chairmanship of Louis and Huron. However, the panel was unable to publish the results of its investigation because of the numerous benefits of the use of the standard for the indirect calibration of another method. All that need be done is to construct a calibration curve based on the measurement of a small series of normal blood samples by the standard and use the method which appears to offer the best one. The panel was unable to publish the results of its investigation because of the numerous benefits of the use of the standard for the indirect calibration of another method. All that need be done is to construct a calibration curve based on the measurement of a small series of normal blood samples by the standard and use the method which appears to offer the best one. The panel was unable to publish the results of its investigation because of the numerous benefits of the use of the standard for the indirect calibration of another method. All that need be done is to construct a calibration curve based on the measurement of a small series of normal blood samples by the standard and use the method which appears to offer the best one.
How should Flow Cytometric LDTs be validated?

- Accuracy
- Specificity
- Sensitivity
- Precision
- Linearity
- Carryover
- Reportable range
- Reference range
- Stability
Cell-based Fluorescence Assays
Laboratory Designed Assay Validation

Soluble analyte assay

- Determine single value
- Use stable reference material
- “Limit of blank” determined by omitting solute
- Precision determined by repeat testing
- Linearity performed by mixing solutions

Cell-based fluorescence assay

- Measure thousands of unique cellular parameters
- No stable reference material available
- Variable cell background / autofluorescence
- Repeat testing limited by short-lived cell viability
- Linearity requires cells/beads of varying fluorescent intensity
Interpretation?
Chronic Lymphocytic Leukemia (CLL)
Features used to recognize CLL

1. B-cell lymphoproliferative disorder (B-LPD)
2. CLL versus other B-LPD:
   a. Matutes score:
      • CD5+, CD22 (dim +/-), SmIg (dim +/-), CD23+, FMC7-
      • (score 4 or 5 = 87% CLL, 0.3% others)
      • (score 0 or 1 = 0.4% CLL, 89% others)
   b. Improved score (Moreau):
      • CD79b (SN8) instead of CD22
      • (score 4 or 5 = 89.3% CLL, 0.3% others)
      • (score 0 or 1 = 0.0% CLL, 69.9% others)
   c. Lower levels of CD19 and CD20 in CLL (Ginaldi)

Matutes E et al. Leukemia 8(10); 1640-5: 1994
Features used to recognize CLL

- CD5+ B-cells
- CD20 dim+
- Smlg dim+/-
- CD23+
Assay Design & Optimization

Goals of the flow cytometric assay:

• Identify all B-cells, even those CD20 dim+/-:
  – CD19

• Determine if B-cells CD5+:
  – distinguish dim+ from background

• Determine if CD20 dim+/-:
  – Other B-cells reproducibly intermediate-to-bright

• Determine if Smlg dim+/-:
  – Other B-cells reproducibly intermediate-to-bright

• Determine if B-cells CD23+:
  – distinguish dim+ from background
Assay Design & Optimization

- Optimize Instrument
- Select Antibody / Fluorochrome combinations:
  - Select “bright” fluorochromes for “dim” signals
  - Avoid issues with data spreading
  - Anticipate issues with tandem breakdown
- Optimize reagents:
  - Confirm antibody specificity and avoid non-specific phenomena
  - Titrate to saturation / maximize signal to noise
- Design analysis strategy
- Establish interpretation and reporting guidelines
Distinguishing dim+/- versus +

Antibody / Fluorochrome selection:
- Sufficient dynamic range between negative and normal B-cell intensity (analytical sensitivity)
- Internal control B-cells confirm expected staining of B-cells
- Most internal control T-cells indicate expected lack of staining
- Determine signal:noise ratio
Which is the brightest CD19 signal?

A. 

B. 
Which is the brightest CD19 signal?

A. CD19 Median Fluorescence Intensity = 9,319

B. CD19 Median Fluorescence Intensity = 25,834
Which is the brightest CD19 signal?

A. CD19 B MFI / T MFI = 176

B. CD19 B MFI / T MFI = 159
Which is the brightest CD19 signal?

**A.**

CD19 B MFI - T MFI / 2 x rSD = 35

**B.**

CD19 B MFI – T / 2 x rSD = 26
Determine if B-cells CD5+

Antibody / Fluorochrome selection:
• CD19 PE-Cy7 adequate intensity to identify all B-cells?
• CD5 PerCP-Cy5.5 adequate intensity to distinguish CD5 positive B-cells from CD5 negative B-cells (analytical sensitivity)?
• Any potential issues with data spreading?
Data Spreading

- Spreading of the negative due to measurement errors after compensation arising from multiple fluorescences spilling into each detector
- Dependent on instrument, configuration & performance, and reagents
- Can be predicted for any pair of detectors
- Should be considered during reagent selection to avoid limiting sensitivity

Nguyen et al., Cytometry Part A 83A; 306-315: 2013
Data Spreading

- Spreading of the negative due to measurement errors after compensation arising from multiple fluorescences spilling into each detector
- Dependent on instrument, configuration & performance, and reagents
- Can be predicted for any pair of detectors
- Should be considered during reagent selection to avoid limiting sensitivity

Nguyen et al., Cytometry Part A 83A; 306-315: 2013
Non-specific phenomena

- Complement C1q mediates interactions between mouse IgG2 class antibodies
- Prevented by either complete serum removal or use of alternate IgG1 clones
- Potential source of artifact in whole blood lysis methods

Antibody Titering

- Use same conditions (volumes) as final assay
- Best signal to noise ratio
- Saturation desirable

Courtesy Paul Wallace, Roswell Park Cancer Institute
Assay Performance Validation

Qualitative Assay:
- Accuracy: comparison with expected results
- Specificity: analytical & clinical specificity = TN/(TN+FP)
- Sensitivity: analytical & clinical sensitivity = TP/(TP+FN)
- Precision: replicates of positive and negative
- Linearity
- Carryover
- Reportable range
- Reference range
- Stability: specimen and processed specimen
Assay Performance Validation

• Clinical accuracy, specificity and sensitivity:
  – Comparison of new assay versus another method (another lab., previous flow assay or morphology/IHC)
  – 20 specimens no-LPD, including PB, BM, LN, other
  – 10 specimens CLL, including PB, BM, LN, other
  – 10 specimens other LPD including PB, BM, LN, other
  – Additional specimens if unexpected results, different anticoagulants

• Precision:
  – 3 replicates 3-5 LPD & 3-5 no-LPD (intra- and inter-assay)
  – compare percent different populations (CV<10%)

• Stability (specimens, processed specimens, reagents):
  – Time points (<1hr, 24 hrs., 48 hrs., or as determined by process)
  – Must fall within precision

• Carryover: run no-LPD, followed by LPD, then no-LPD
Population with CLL-like phenotype, but only small percent of total events.

Clinical significance?
Monoclonal B Lymphocytosis

- Monoclonal B-cell population
  - persisting for at least 3 months
  - total B-cell count below $5 \times 10^9$/L
  - no other features diagnostic of a B-cell lymphoid neoplasm
- CLL-like and non-CLL-like MBL
- Prevalence CLL-like MBL proportional to assay sensitivity
- Clinical significance related to absolute count: evaluation of lymphocytosis vs. population studies
- Higher count CLL-like MBL:
  - progression to CLL 1% per year
  - decreased normal B-cells leading to increased infection
MBL vs. CLL
< 5 x 10⁹/L?
MBL vs. CLL?

- 20 peripheral blood specimens with CLL-like B-cells
- Results from single platform (SP) tube compared with 2 dual platform (DP) tubes:
  - Kappa, lambda, CD5, CD10, CD19, CD38, CD45, CD20
  - FMC-7, CD23, CD19, CD5
- 3 discordant results relative to threshold of $5 \times 10^9$/L

<table>
<thead>
<tr>
<th>Case</th>
<th>Single Platform</th>
<th>Dual Platform</th>
<th>Clinical Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tube A</td>
<td>Tube B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rack</td>
<td>No-R</td>
</tr>
<tr>
<td>1</td>
<td>*</td>
<td>5.66</td>
<td>3.22</td>
</tr>
<tr>
<td>2</td>
<td>*</td>
<td>5.69</td>
<td>4.59</td>
</tr>
<tr>
<td>3</td>
<td>*</td>
<td>4.36</td>
<td>5.67</td>
</tr>
</tbody>
</table>

Rollins-Raval et al., Cytometry Part B 84B; 149-156: 2013
MBL vs. CLL?

Rollins-Raval et al., Cytometry Part B 84B; 149-156: 2013
Assay Performance Validation

Quasi-quantitative Assay:
- Accuracy: comparison with expected results
- Specificity: analytical & clinical specificity = TN/(TN+FP)
- Sensitivity: analytical & clinical sensitivity = TP/(TP+FN)
- Precision: replicates of positive and negative
- Linearity
- Carryover
- Reportable range
- Reference range
- Stability: specimen and processed specimen

Minimal Residual CLL?

CD5+ T-cells

CD5+ B-cells
Minimal Residual CLL

• Evolving treatment of CLL:
  – Prognostic markers identify higher-risk patients
  – Highly effective immunotherapy can eradicate MRD < 0.01%, or 0.001%
  – MRD-negative patients have a survival advantage
  – Linear increase in progression free survival per log tumor depletion (10 – 0.001 x 10^9/L = 4 x 1.5 years)

• Improved detection of MRD:
  – Molecular
  – Flow cytometric
CLL-MRD detection using CD19 / CD5 / kappa / lambda

• CD5+ B-cells:
  – Normal B-cell subset (typically <30% B-cells)
  – Increases with regeneration, up to 90%
  – Many CLL-cells have only weak expression

• Light chain restriction:
  – Highly skewed K:L ratio following therapy
  – Accuracy of ratio limited by only a few events
  – Confounded by admixed polytypic B-cells
CLL-MRD detection using CD19 / CD5 / kappa / lambda

[CD5+ B-cells > 82% & K:L ratio <0.005:1 or > 32:1]

• Positive predictive value 100%:
  – 47% of cases (72% with >1% CLL, 37% with <1%)
• Negative predictive value poor:
  – MRD+ in 42% with normal K:L ratio
  – MRD+ in 31% with <10% CD5+ B-cells
• Qualitative only

AC Rawstron et al., Leukemia 27; 142-149: 2013
CLL-MRD Assay Design & Optimization

• Informative antibodies:
  – CD20 single marker with best separation
  – Include CD5, CD20 and CD79b = 10x higher sensitivity cf. CD19 / CD5 / kappa / lambda
  – CD5/CD19, CD20/CD38, CD81/22, CD79a/CD43 lowest inter-laboratory variation and false +
• Fluorochrome antibody combinations
• Number events = 500,000 (0.01% = 50 events)
• Analysis strategy

AC Rawstron et al., Leukemia 2; 956-964: 2007
CLL-MRD Assay Design & Optimization

CD19, CD20, CD22, CD81, CD79b, CD5, CD43, CD3
Platform-independent reagent specification

Preferred CD20 relative fluorescence intensity
MFI B-cells / MFI T-cells >20

Rawstron AC et al on behalf of ERIC manuscript in preparation
CLL MRD
Assay Performance Validation

Quasi-quantitative Assay:
• Accuracy
• Specificity
• Sensitivity:
  – Analytical: limit of detection (LOD) / limit of blank (LOB)
  – Functional: lower limit of quantitation (LLOQ)
• Precision
• Linearity
• Carryover
• Reportable range
• Reference range
• Stability

CLL MRD
Assay Performance Validation

Dilutional studies:
• Verify desired target threshold (0.01% or 0.001%)
• Use WBC’s from 5 specimens with CLL spiked at different levels into 5 normal specimens
• Assay over 24 hours, in 5 replicate and divide data into 5 files:
  – Lower limit of blank: 95% negatives < target
  – Lower limit of detection: 95% low positives > target
  – Lower limit of quantitation: lowest level where replicates reliably meet criteria for precision
  – Linearity across reportable range
  – Stability
CLL dilutional study

1% CLL

0.1% CLL

0.01% CLL
How should Flow Cytometric LDTs be validated?

• More difficult than for many clinical laboratory tests
• Start by deciding on the purpose of the test:
  – Qualitative, Quasi-quantitative, Quantitative
• Develop & optimize assay to meet diagnostic needs
• Stop making changes!
• Follow ICSH-ICCS guidelines for validation of cell-based fluorescence assays