



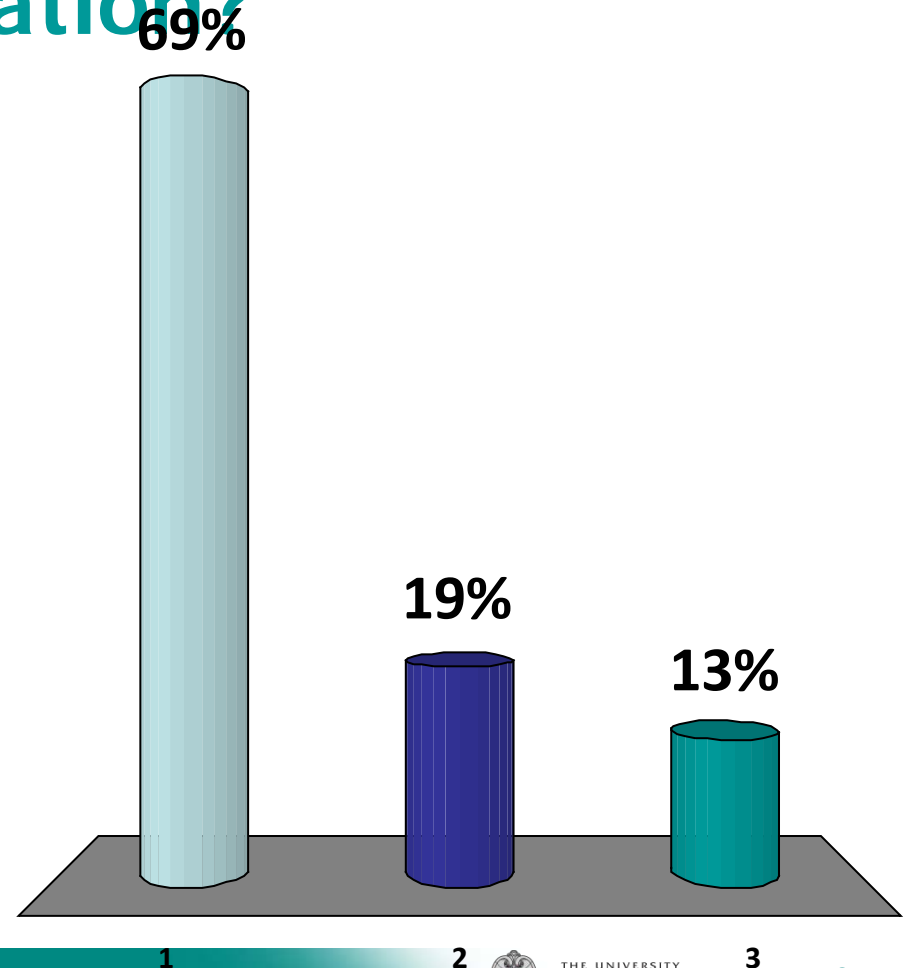
Validation and Verification--- or Verification and Validation of Molecular Assays



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Diseases

Does your laboratory have a policy and procedure for method validation?

1. Yes
2. No
3. If we do, I've never seen it



FDA-Cleared TB Molecular Assays

**Amplified *M. tb* Direct Test®
(MTD): Gen-Probe, Inc.**

**Amplicor® *M. tuberculosis*
(MTB): Roche Diagnostics**

Non-FDA Cleared Molecular Tests

- Commercial tests available
 - **BD ProbeTec™ MTB Direct Detection**
 - **COBAS® Amplicor® MTB Test**
 - **COBAS® TaqMan® MTB Test**
 - **Hain Line Probe Assay**
 - TB detection and molecular resistance
 - **Innogenetics INNO-LiPA**
 - **Cepheid GeneXpert®**
 - TB Detection and rifampin resistance
- Laboratory-Developed Tests (Home brews)
- Off-label use of FDA-cleared tests

CLIA Requirements

- After April 24, 2003, any new high complexity test introduced into the laboratory must be verified
 - Laboratory developed test
 - Modification of the manufacturer's test procedure (e.g. different specimen type)
 - Any non-FDA cleared method

Terminology or

"What's in a name? That which we call a rose
By any other name would smell as sweet."



- Validation
 - The documentation that a test which has already been **verified** is repeatedly giving the expected results as the test is performed over a period of time.
 - **Quality control**
 - **Proficiency testing**
 - **Validation of employee competency**
 - **Instrument calibration**
 - **Correlation with clinical findings**

Terminology

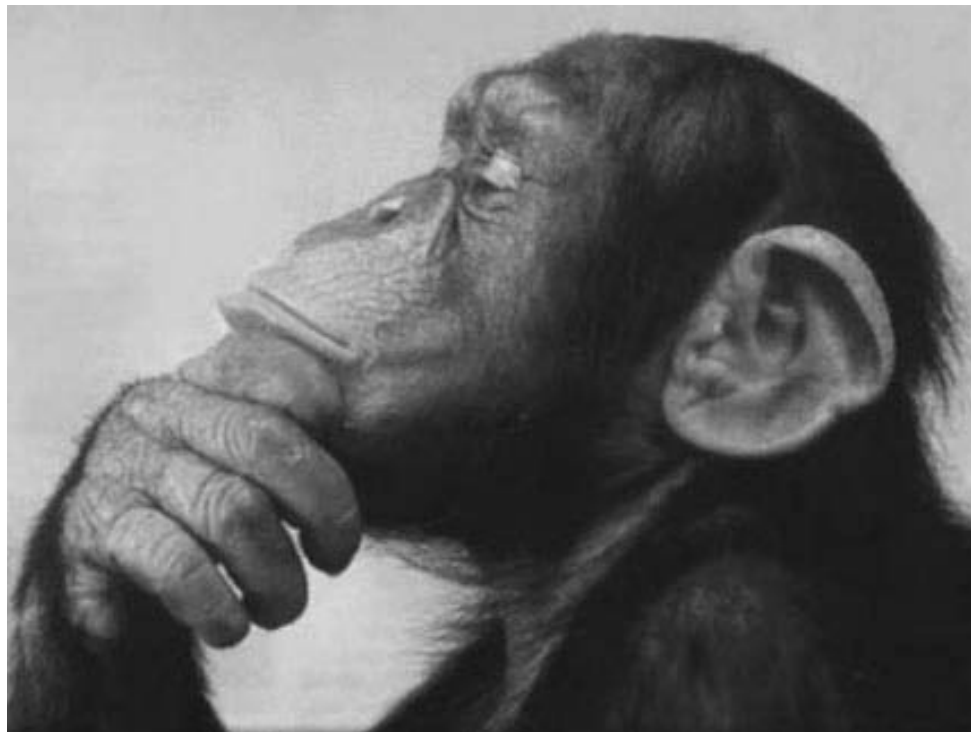


- Verification
 - The documentation of either commercial or laboratory developed tests to determine or confirm test performance characteristics before the test system is used for patient testing
 - A one-time process

Purposes of Method Verification

- To quantifiably characterize test performance
- To assess the potential for error
- To identify method-to-method differences
- To meet regulatory guidelines

Verification Study Design for Laboratory Developed Tests (LDTs)



CLIA Performance Characteristics

Section 493.1253

- CLIA specifies performance characteristics---
--but not how to do it or criteria to be used
 - Accuracy
 - Precision
 - Analytical sensitivity
 - Analytical specificity
 - Reportable Range
 - Reference range(s)
 - Any other characteristics required for test performance and interpretation of results

Verification Study Design

- Establish the type and number of specimens necessary
- Decide on a comparative method or “gold standard”
- Acceptance criteria
- Methods for resolving discrepancies

Discrepant Analysis

- Discrepancies
 - May arise due to errors in the method being evaluated
 - Comparative method is not 100% accurate
- Resolving discrepancies
 - Use a designated reference standard
 - Send to another laboratory that uses a different method
 - Use a test that targets another area of the gene

What Types of Samples can be used?

- Should be typical of those that will be routinely tested
 - Patient samples with known results
 - Retention specimens
 - Specimens from another laboratory
- Other Options
 - Quality control material
 - Proficiency testing samples
 - Calibration material
 - Spiked negative patient specimens
 - Manufacturer's verification panels???

Analytical Accuracy

- Closeness of an individual measurement to the “true” value, as determined by a reference method
 - Synonymous with test efficiency
- Numbers of known positive and negative specimens should be balanced or statistically significant to have confidence in the test result
 - e.g. ≥ 50 positive, ≥ 50 negative
 - Confidence interval of 78-97% (CLSI EP12)

$$\frac{\text{Number of correct results}}{\text{Total number of results}} \times 100$$

Side-by-Side Comparison of Real-Time PCR Assay to Gen Probe® Amplified™ MTD Test at WSLH

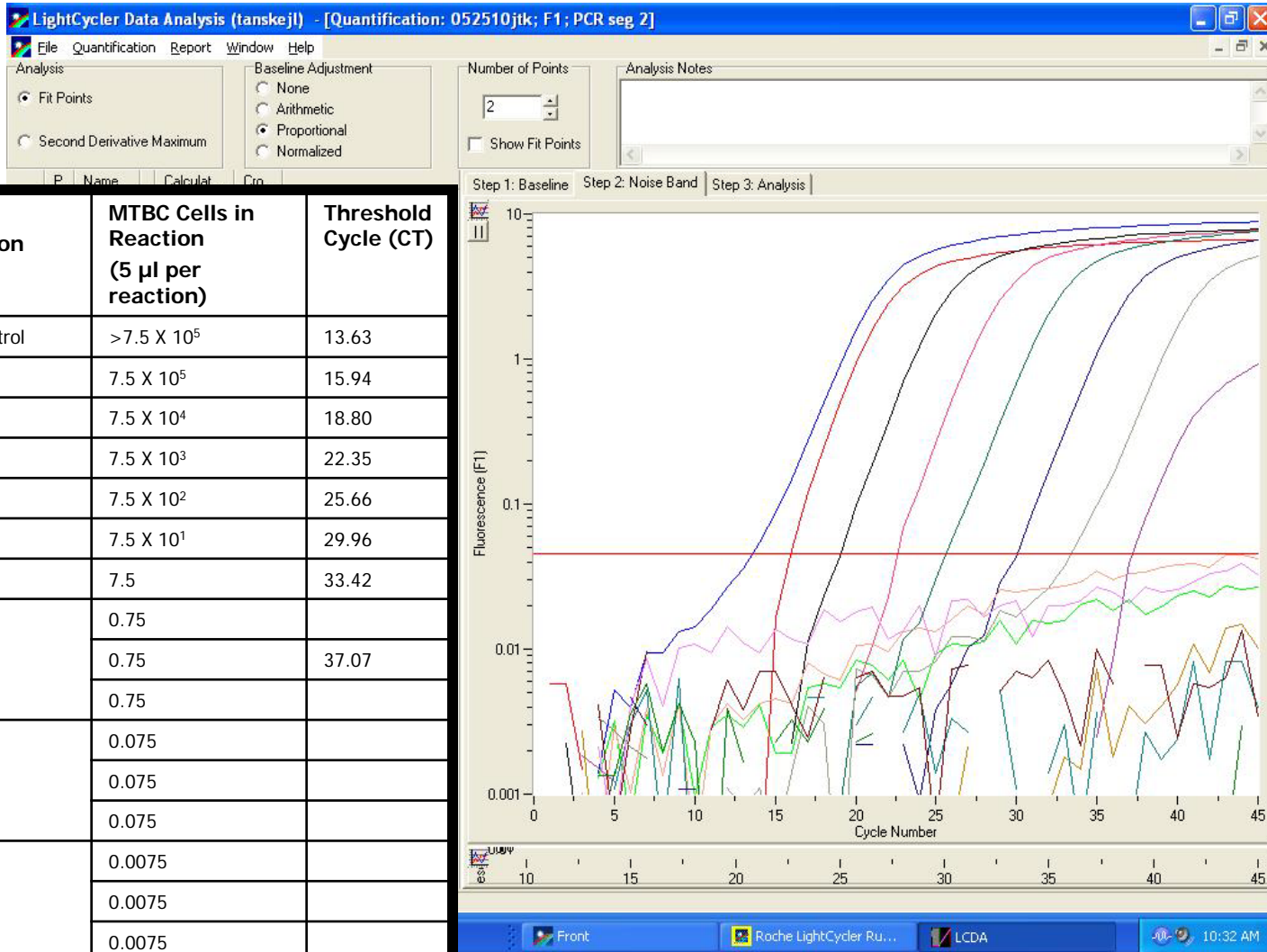
Number of Specimens	MTD Positive	MTD Negative	MTD Inhibited
Real-Time PCR Positive	25	0	2
Real-Time PCR Inhibited	0	0	0
Real-Time PCR Negative	0	34	3

Analytical Sensitivity (Limits of Detection)

LoD---the lowest amount of target that can be detected by a test system with a stated probability

- **Methods**
 - Spiking whole organisms into negative specimens
 - Known CFUs/ml, PFUs/ml, TCID₅₀/ml
 - Spiking with known number of copies of the target
- **Evaluate for the influence of microbial diversity**
 - Different patient isolates, serogroups, serotypes, lineages, resistance phenotypes, etc.
- **Reference---EP17-A: Protocols for Determination of Limits of Detection and Quantitation**

Sensitivity

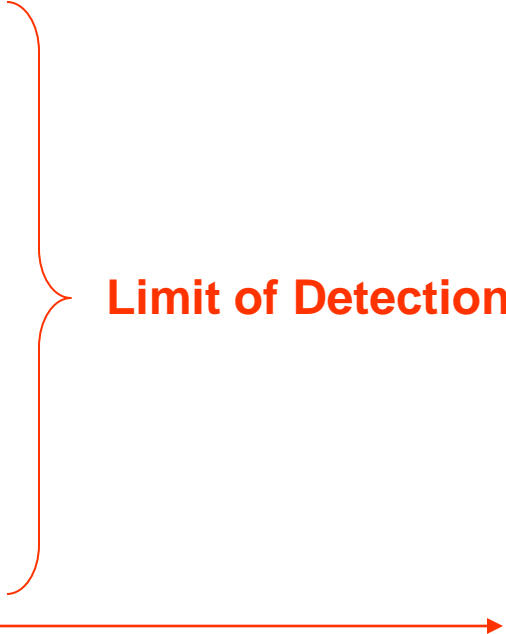


Sensitivity

Cell Suspension	Cells/Reaction	Threshold Cycle
1.5 X 10 ³	7.5	32.98
	7.5	33.57
	7.5	33.81
	7.5	32.89
	7.5	32.96
	7.5	32.5
Average		33.11
1.5 X 10 ²	0.75	36.47
	0.75	>40
	0.75	35.7
	0.75	35.7
	0.75	34.38
	0.75	35.77
	0.75	38.38
	0.75	35.88
	0.75	36.86
	0.75	36.51
	0.75	>40
	0.75	36.08
Average		36.17
1.5 X 10 ¹	0.075	>40
	0.075	37.17
	0.075	>40
	0.075	>40
	0.075	>40

Limit of Detection

Cut-off Threshold cycle for positive



Analytical Specificity

Ability of a method to detect only the analyte that it was designed to measure

- Verify for cross-reactivity
 - Organisms that are closely related to the target organism
 - Organisms that represent normal flora of the specimen being tested
 - Organisms that cause similar disease syndromes
- Can use whole organisms that go thru the complete method and/or extracted nucleic acids

Specificity and Breadth of Detection

Negative Real-Time PCR Result	Positive Real-Time PCR Result
<p><i>M. avium</i> complex ATCC700898</p> <p><i>M. gordonae</i> ATCC 1218</p> <p><i>M. xenopi</i></p> <p><i>M. scrofulaceum</i></p> <p><i>M. peregrinum</i> ATCC 700686</p> <p><i>M. smegmatis</i> ATCC 1546</p> <p><i>M. marinum</i></p> <p><i>M. mucogenicum</i></p> <p><i>M. chelonae</i></p> <p><i>M. abscessus</i></p> <p><i>M. fortuitum</i> ATCC 1447</p> <p><i>M. kansasii</i> ATCC 12478</p>	<p><i>Tsukamurella</i> sp.</p> <p>MTBC ATCC 27294</p> <p><i>L. pneumophila</i></p> <p>MTBC ATCC 35828</p> <p><i>N. meningitidis</i></p> <p><i>S. Pneumoniae</i></p> <p>32 TB patient isolates and 3 <i>M. bovis</i> BCG isolates</p> <p>Group A <i>Streptococcus</i></p> <p><i>H. influenzae</i></p> <p><i>P. aeruginosa</i></p> <p><i>K. pneumoniae</i></p> <p><i>B. Pertussis</i></p> <p><i>B. parapertussis</i></p>

Analytical Specificity con't

- Other reasons for non-specific (false positive) results
 - Target or amplicon cross- contamination
 - High background noise
 - Other sources of nonspecific signal
- Test for cross-contamination
 - Testing negative specimens alternated with strong positive specimens in the same run

Precision (Reproducibility)

- Within run
- Run-to-run, same day
- Day-to-day, different analysts
- Inter-instrument
- Protocol described in CLS EP12
 - e.g. For a qualitative assay interpreted from a quantitatively measured signal-----Calculate SD and CV from 10-20 day-to-day quality control results
 - Run aliquots of a single specimen 30 times in a single run
 - Panel of samples run by different analysts
- Goal of 95% typical of PCR assays

Reportable Range

- For qualitative assay
 - Detected or not detected
- For quantitative assay
 - The range of results for which a test has been proven to yield numerically accurate results. (CLSI Document EP17-A)

Verification Documentation

- Write up
 - Purpose and Background
 - Methods
 - Results
 - Conclusions
- Sign off and approval by Laboratory Director or designee who qualifies as a Director
- Save for ≥ 2 years after test is discontinued

Comparison of Real-Time PCR and Gen Probe MTD to Culture

		Culture Results			Performance	
		Positive	Negative	Total	Sensitivity	Specificity
Real-Time PCR	Positive	27	0	27	27/29 (93.1%)	100%
	Negative	2	35	37		
	Inhibited	0	0	0		
	Total	29	35	64		
Gen Probe MTD	Positive	25	0	25	25/29 (86.2%)	100%
	Negative	2	32	34		
	Inhibited	2	3	5		
	Total	29	35	64		

FDA-Cleared Test Verification

- If unmodified
 - Accuracy
 - Precision
 - Reportable range
 - Reference range
- Don't have to assess sensitivity and specificity
- Accuracy and precision should fall within manufacturers specifications
 - Can use fewer specimens

Public Health Laboratory Exceptions

- Exceptions for PHLs when calibration or control materials are not available
 - e.g. During public health emergencies, tests for emergent diseases, or public health threats
 - Emergency Use Authorization (EUA)
- Given temporary CLIA exemption
 - Must follow CDC protocols w/o modification

Public Health Laboratory Exceptions con't

- Given temporary CLIA exemption
 - Must follow CDC protocols w/o modification
 - Personnel must show proficiency in the test method
 - Must document alternative methods to show accuracy
 - Send a number of samples to CDC for verification
 - Test with another method
- Must do complete validation when calibration material is available or EUA expires

Summary

- Verification studies are a “Necessity of Laboratory Life”
- Verification studies require a major time commitment
- Verification studies can be a major expense
- Verification studies must be carefully designed
 - Important to utilize the guidelines that are available

References

- CLSI document MM3-A2. Molecular Diagnostic Methods for Infectious Diseases.
- CLSI document MM6-A. Quantitative Molecular Methods for Infectious Diseases.
- CLSI document EP12-A2. User Protocol for Evaluation of Qualitative Test Performance.
- CLSI document EP17-A. Protocols for Determination of Limits of Detection.

References

- CLSI EP05-A2 document. Evaluation of Precision Performance of Quantitative Measurement Methods.
- CUMITECH 31. Verification and Validation of Procedures in the Clinical Microbiology Laboratory. ASM Press
- National Laboratory Training Network Verification of Infectious Disease Molecular Assays. August 2005. Copies available www.nltn.org



Thank you!