Verification of Procedures in the Molecular Diagnostic Laboratory

Jeffrey P. Massey, Dr.P.H., HCLD(ABB)
Manager, Molecular Biology Section
Michigan Department of Community Health
Masseyj@michigan.gov

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Difficulties with Verification of Molecular Assays

- Lack of samples for testing (calibration, QC, or PT material)
- Molecular assays often measure a different target
- Molecular assays are more sensitive than the current "Gold Standard"

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Another Problem....

- CLIA regulations state <u>what</u> must be done, but don't give any specifics on <u>how</u> it must be done.

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Accuracy

- The lab must document how it determined the new method produces accurate results based upon:
  - Clinical sensitivity
  - Clinical specificity
  - Positive predictive value
  - Negative predictive value

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Accuracy

- Verify the method produces the correct result (test reliabilitity)

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

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How to Demonstrate Accuracy

Accomplished by:

- Test reference materials
- Compare test results vs. reference method (shown to provide clinically valid results)
- Compare split sample results

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Samples for Accuracy Study

- Patient samples with known result
- QC material
- PT material
- Calibration material

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Precision

- Measure of the extent that repeat testing is in agreement (reproducibility)

$$\frac{\text{\# of repeated results in agreement}}{\text{total number of results}} \times 100\%$$

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Precision

- The lab will document how it evaluated:
  - Day-to-day variance
  - Run-to-run variance
  - Within-a-run variance
  - Operator variance

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How to Demonstrate Precision

- Repeat testing of known patient samples over time
- Test QC samples in duplicate and over time
- Repeat testing of calibration materials over time

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Analytical Sensitivity

- Measure of the smallest quantity of an analyte that is reproducibly distinguished from background

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Analytical Sensitivity

- The lab will document the lowest concentration or amount of the analyte that can be consistently measured.
  - Minimum detection limits (how much analyte must be present to be consistently measured)

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How to Establish Analytical Sensitivity

- Dilutions of virus or bacteria of known quantity
  - Pfu, ID_{50}, direct quantitation by EM
- Quantitate amount of RNA or DNA extracted
- Control material of known concentration or copy number

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Analytical Specificity

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

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Analytical Specificity

- The lab will document that the procedure will measure only the analyte intended to be measured
- The lab will also document interfering substances

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What should be tested?

- Closely related organisms
- Organisms that may be present from the same site
- Organisms that may cause similar symptoms

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Analytical Specificity: Interfering Substances

- Document information regarding interfering substances from product information, literature, or own testing
  - Specimen hemolysis
  - Anticoagulant
  - Lipemia
  - Specimen type (stool, urine, etc.)

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Quality Systems: Control Procedures (493.1256(3)(iv-v))

- Each day of testing:
  - For tests with extraction phase, 2 control materials. One must detect errors in extraction process
  - Molecular amplification procedure: 2 control materials and another to detect inhibition (if inhibition is a significant source of false negative results)

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Reportable Range

- Establish the upper and lower limits of the test system
- The lab will document how the reportable range of patient test results was established

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Reference Range

- If the procedure is a quantitative assay, the lab will document how the normal range was established
- Reference range must be appropriate for the lab's patient population
  - Reflects type of specimen and demographics (e.g., age and sex)

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Conducting a Verification Study

- Planning
- Testing
- Analyze data & resolve discrepancies

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Conducting a Verification Study #1

- Planning
  - Determine the number and type of specimens
  - Establish acceptance criteria
  - State the methods to resolve discrepancies

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How Many to Test? Suggestion #1

- At least 1 week parallel testing with existing method (minimum 50 samples)
- Example: when converting from standard PCR to Real-time PCR

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Suggestion #2

- Test known representative samples (12 –15 common isolates, stock cultures or clinical samples): total of 50 or more tests
- Example: Implementing a new molecular assay. Use samples confirmed as present or absent by another method

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Suggestion #3

- Confirm that 20 to 50 organisms agree in concurrent testing with current method or with results of split testing with another lab
- Example: Use when implementing a method already in use by another lab

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Establish Acceptance Criteria

- A general rule of thumb is 95% agreement
- This can vary depending on the analyte being tested
- Performance criteria is not stated in the CLIA regulations

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Conducting a Verification Study – Step #2

- Testing
  - Become proficient with the assay
    - If FDA test – complete training, personnel competency, etc.
    - If in-house – establish performance characteristics using a small verification panel
- Perform testing on blinded samples

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Evaluation of Non-FDA Approved HSV Assay

- Detection of HSV 1 & 2 by rapid-cycle real-time PCR using FRET
- Amplifications performed on Roche LightCycler
- HSV 1 and HSV 2 differentiated by melting temperature
  - HSV 1 = 54ºC
  - HSV 2 = 68ºC

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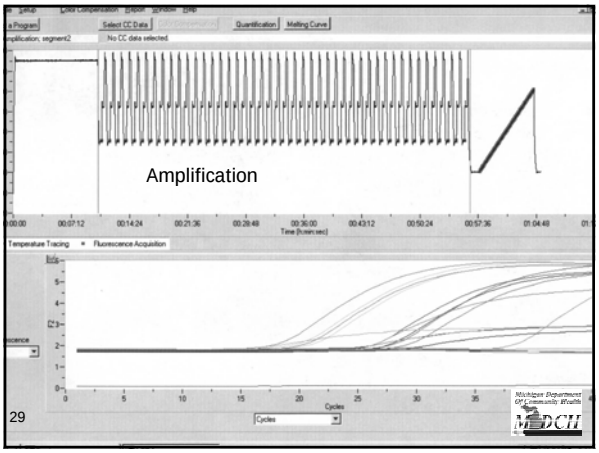

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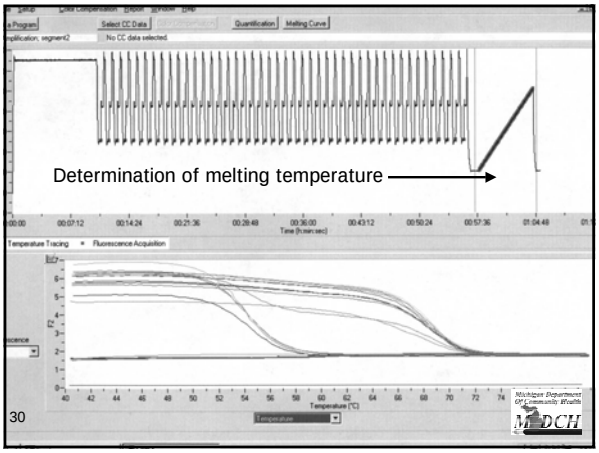
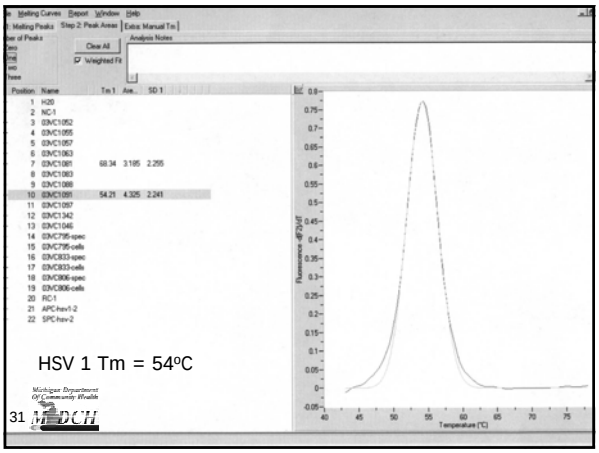
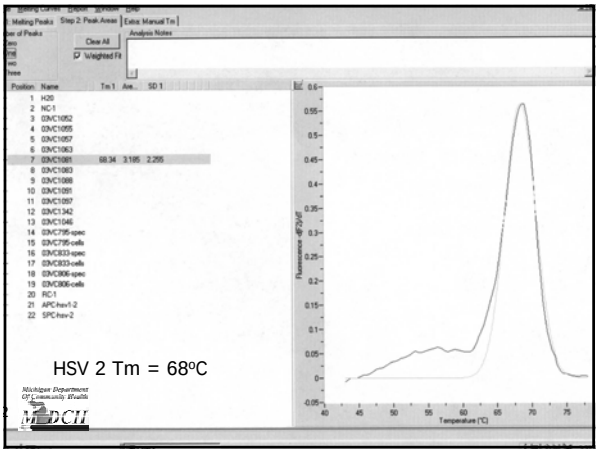
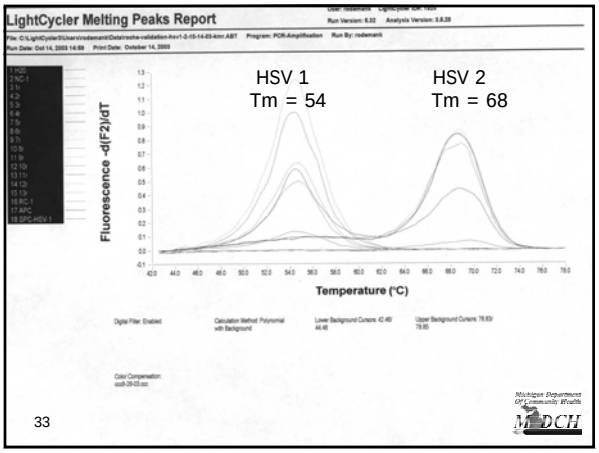


30


HSV 1 Tm = 54ºC
Temperature (°C)


HSV 2 Tm = 68ºC
Temperature (°C)


LightCycler Melting Peaks Report
HSV 1
Tm = 54
HSV 2
Tm = 68
Fluorescence -d(F2)/dT
Temperature (°C)
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Conducting a Verification Study – Step #3

- Data Analysis & Resolving Discrepancies
  - Calculate accuracy, precision, reportable range, and reference range (and analytical sensitivity & specificity if in-house assay)
  - Resolve discrepancies

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How to Resolve Discrepancies

- Sequence amplicon
- Determine clinical status of patient
- Test samples by another lab

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Summary of Results

	Neg	PCR HSV1	PCR HSV2	Total
Culture Neg	9	0	1	10
Culture HSV 1	0	8	1	9
Culture HSV2	0	0	11	11

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Ct and Tm Data

	Culture	#	Ct	Tm
HSV-1	9	8	26.5	54ºC
HSV-2	11	11	25.8	68ºC

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Summary of Performance Characteristics

	Actual Results	Lab Goal
Accuracy	95.2%	95%
Precision	100%	95%
Analytical Sensitivity	6 viral particles	10 viral particles
Analytical Specificity	VZV(-), EBV(-), CMV(-)	No cross reactions

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Evaluation of Non-FDA Approved HSV Assay

- Detection of HSV 1 & 2 by rapid-cycle real-time PCR using FRET
- Amplifications performed on Roche LightCycler
- HSV 1 and HSV 2 differentiated by melting temperature
  - HSV 1 = 54ºC
  - HSV 2 = 68ºC

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Summary of Evaluation Study

- Accuracy and precision within predetermined range of acceptability
- Excellent correlation results with cell culture
- Acceptable lower limit of detection
- No cross reactivity with other viruses
- Distinctive differences in melting temperatures: 54°C vs. 68°C

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Final Step - Implementation

- Review and approval by Lab Director
- Maintain all documentation for 2 years
- Lab report to incorporate ASR disclaimer as needed
- Establish twice a year verification of assay (in place of 3X PT)
- Assure ongoing QA

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References

- The CLIA regulations, www.phppo.cdc.gov/clia/default.asp
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- Cumitec 31, Verification and Validation of Procedures in the Clinical Microbiology Laboratory, http://www.asmpress.org/browse/bound/index.asp?SelectedItem=10122
- CAP molecular pathology checklist, http://www.cap.org/apps/docs/laboratory_accreditation/checklists/checklistftp.html
- NCCLS document MM3-A, Molecular Diagnostic Methods for Infectious Diseases
- http://www.nccls.org/es/source/orders/free/mm3-a.pdf
- NCCLS document MM6-A, Quantitative Molecular Methods for Infectious Diseases.
- http://www.nccls.org/es/source/orders/free/mm6-a.pdf

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