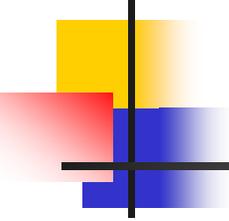




# Verification of Procedures in the Molecular Diagnostic Laboratory

---

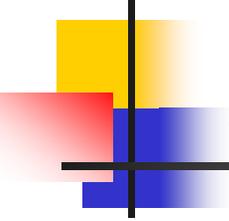
Jeffrey P. Massey, Dr.P.H., HCLD(ABB)  
Manager, Molecular Biology Section  
Michigan Department of Community Health  
[Masseyj@michigan.gov](mailto:Masseyj@michigan.gov)



# Difficulties with Verification of Molecular Assays

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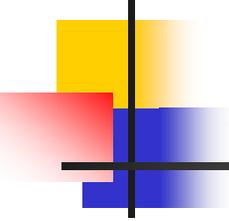
- Lack of samples for testing (calibration, QC, or PT material)
- Molecular assays often measure a different target
- Molecular assays are more sensitive than the current “Gold Standard”



# Another Problem....

---

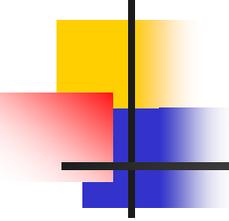
- CLIA regulations state what must be done, but don't give any specifics on how it must be done.



# 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

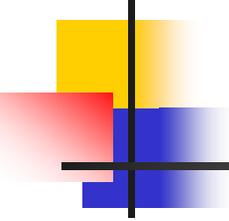


# Accuracy

---

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

Number of correct results x 100%  
Total number of results

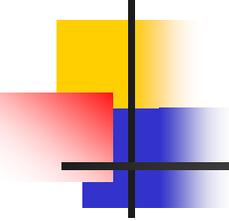


# How to Demonstrate Accuracy

---

Accomplished by:

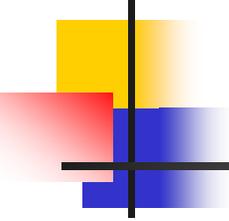
- Test reference materials
- Compare test results vs. reference method (shown to provide clinically valid results)
- Compare split sample results



# Samples for Accuracy Study

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

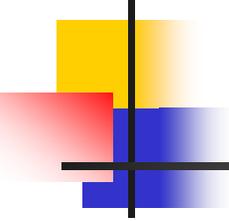


# Precision

---

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

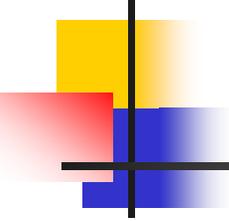
$$\frac{\text{\# of repeated results in agreement}}{\text{total number of results}} \times 100\%$$



# Precision

---

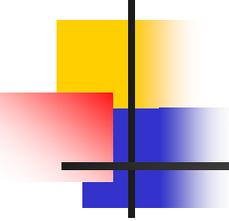
- The lab will document how it evaluated:
  - Day-to-day variance
  - Run-to-run variance
  - Within-a-run variance
  - Operator variance



# How to Demonstrate Precision

---

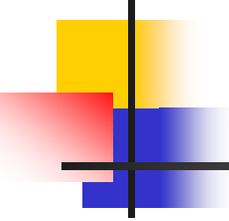
- Repeat testing of known patient samples over time
- Test QC samples in duplicate and over time
- Repeat testing of calibration materials over time



# Analytical Sensitivity

---

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



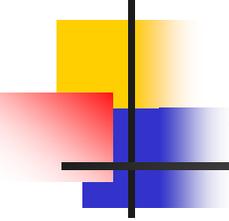
# Analytical Sensitivity

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- 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)

# How to Establish Analytical Sensitivity

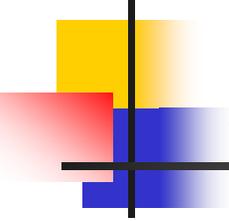
- Dilutions of virus or bacteria of known quantity
  - Pfu, ID<sub>50</sub>, direct quantitation by EM
- Quantitate amount of RNA or DNA extracted
- Control material of known concentration or copy number



# Analytical Specificity

---

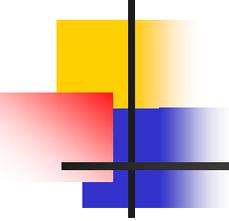
- Ability of a method to detect only the analyte it was designed to measure



# Analytical Specificity

---

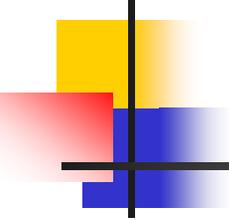
- The lab will document that the procedure will measure only the analyte intended to be measured
- The lab will also document interfering substances



# What should be tested?

---

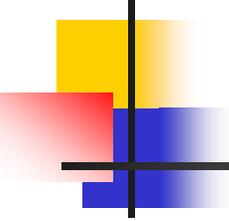
- Closely related organisms
- Organisms that may be present from the same site
- Organisms that may cause similar symptoms



# Analytical Specificity: Interfering Substances

---

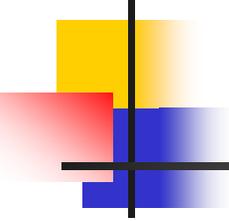
- Document information regarding interfering substances from product information, literature, or own testing
  - Specimen hemolysis
  - Anticoagulant
  - Lipemia
  - Specimen type (stool, urine, etc.)



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

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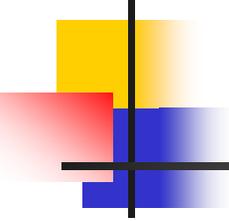
- Each day of testing:
  - For tests with extraction phase, 2 control materials. One must detect errors in extraction process
  - Molecular amplification procedure: 2 control materials and another to detect inhibition (if inhibition is a significant source of false negative results)



# Reportable Range

---

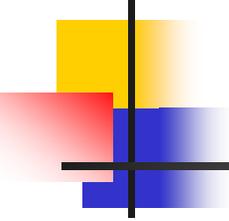
- Establish the upper and lower limits of the test system
- The lab will document how the reportable range of patient test results was established



# 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)



# Conducting a Verification Study

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- Planning
- Testing
- Analyze data & resolve discrepancies

# Conducting a Verification Study #1

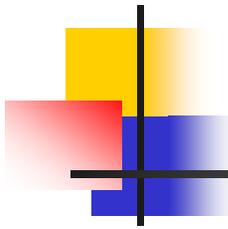
- Planning
  - Determine the number and type of specimens
  - Establish acceptance criteria
  - State the methods to resolve discrepancies

# How Many to Test?

## Suggestion #1

---

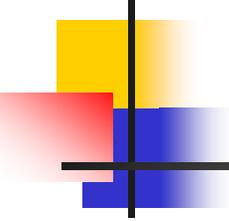
- At least 1 week parallel testing with existing method (minimum 50 samples)
- Example: when converting from standard PCR to Real-time PCR



## Suggestion #2

---

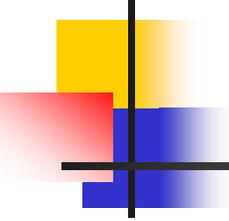
- Test known representative samples (12 –15 common isolates, stock cultures or clinical samples): total of 50 or more tests
- Example: Implementing a new molecular assay. Use samples confirmed as present or absent by another method



## Suggestion #3

---

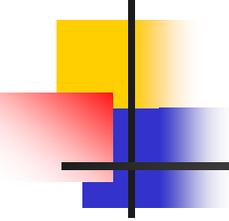
- Confirm that 20 to 50 organisms agree in concurrent testing with current method or with results of split testing with another lab
- Example: Use when implementing a method already in use by another lab



# Establish Acceptance Criteria

---

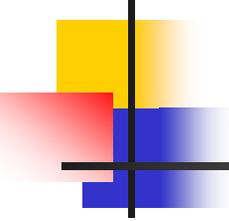
- A general rule of thumb is 95% agreement
- This can vary depending on the analyte being tested
- Performance criteria is not stated in the CLIA regulations



# Conducting a Verification Study – Step #2

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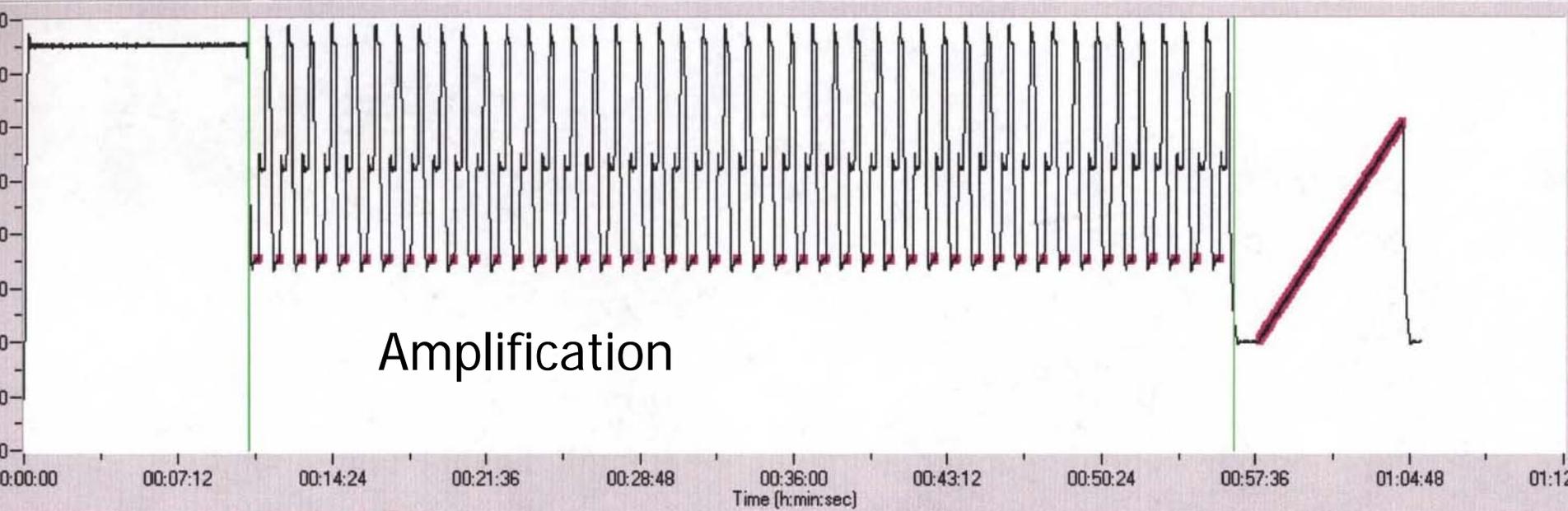
- Testing
  - Become proficient with the assay
    - If FDA test – complete training, personnel competency, etc.
    - If in-house – establish performance characteristics using a small verification panel
- Perform testing on blinded samples



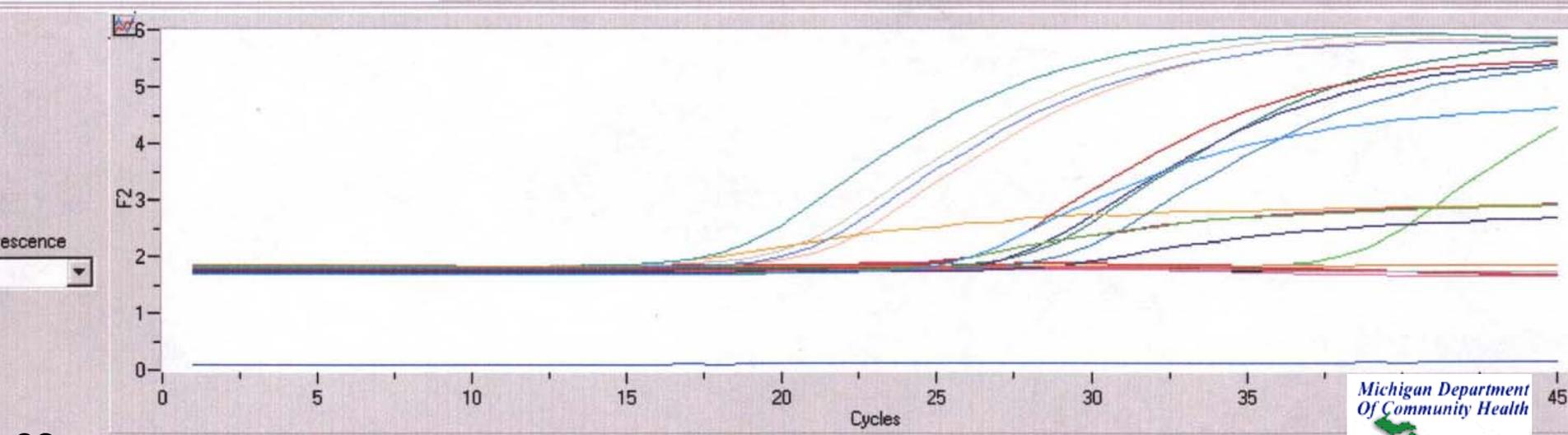
# Evaluation of Non-FDA Approved HSV Assay

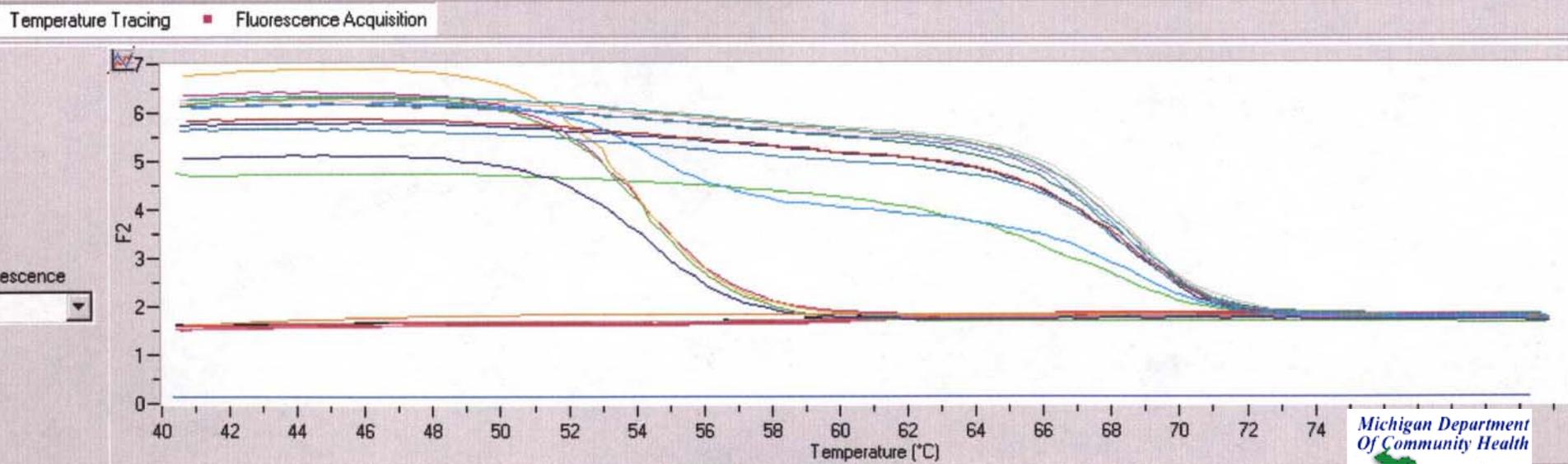
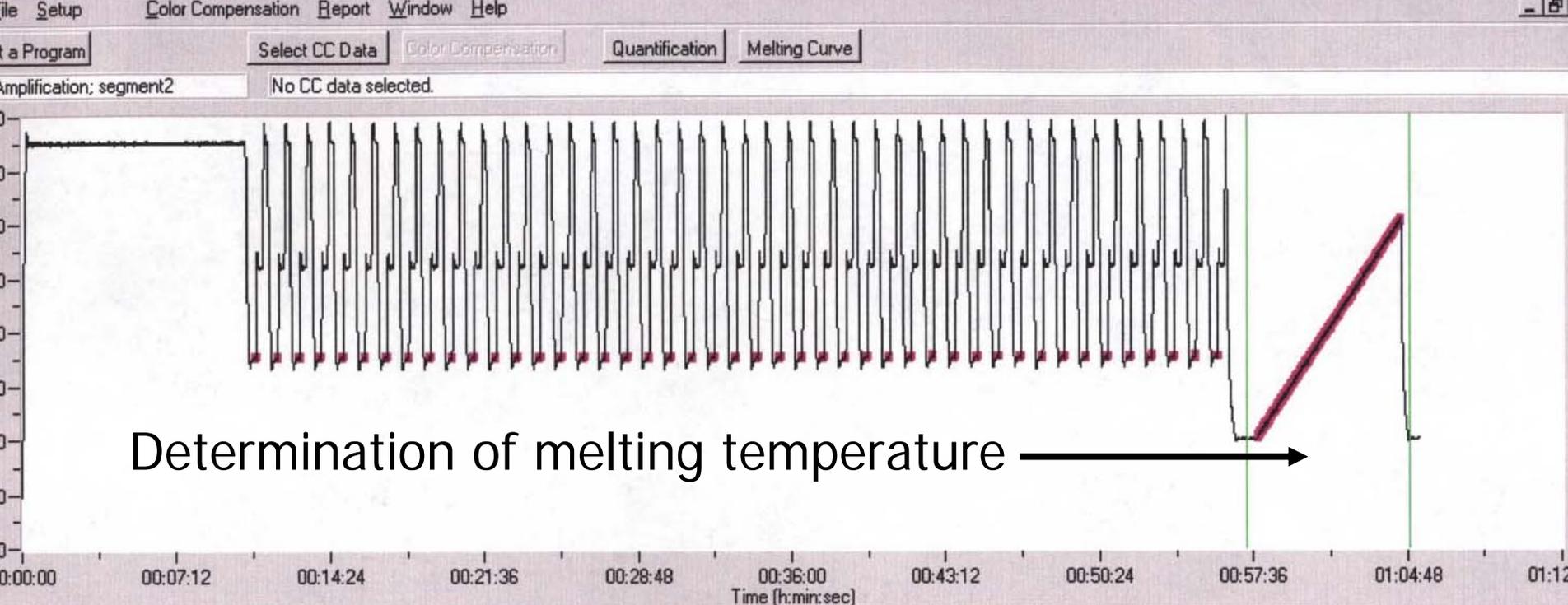
---

- 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 = 54°C
  - HSV 2 = 68°C



Temperature Tracing ■ Fluorescence Acquisition





Number of Peaks

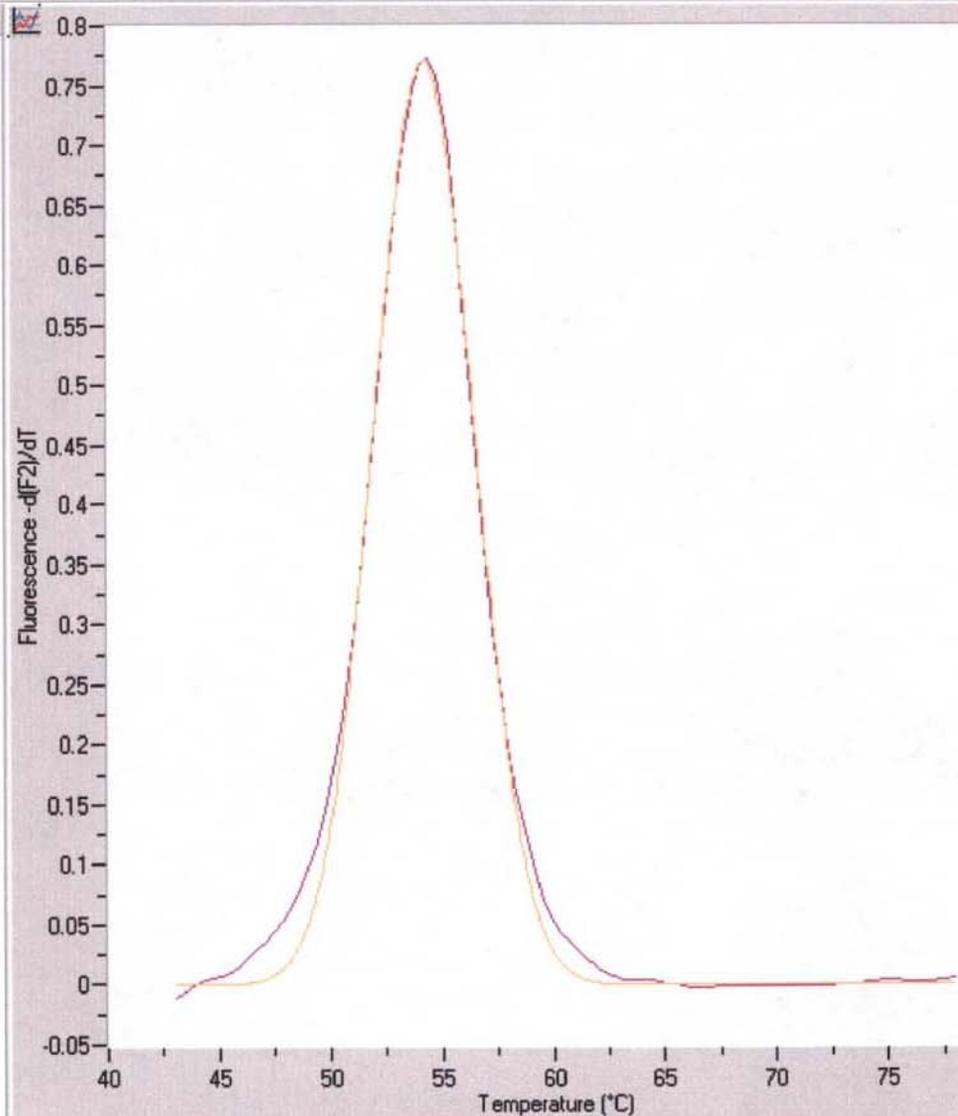
Zero  
One  
Two  
Three

Clear All

 Weighted Fit

Analysis Notes

| Position | Name          | Tm 1  | Area  | SD 1  |
|----------|---------------|-------|-------|-------|
| 1        | H2O           |       |       |       |
| 2        | NC-1          |       |       |       |
| 3        | 03VC1052      |       |       |       |
| 4        | 03VC1055      |       |       |       |
| 5        | 03VC1057      |       |       |       |
| 6        | 03VC1063      |       |       |       |
| 7        | 03VC1081      | 68.34 | 3.185 | 2.255 |
| 8        | 03VC1083      |       |       |       |
| 9        | 03VC1088      |       |       |       |
| 10       | 03VC1091      | 54.21 | 4.325 | 2.241 |
| 11       | 03VC1097      |       |       |       |
| 12       | 03VC1342      |       |       |       |
| 13       | 03VC1046      |       |       |       |
| 14       | 03VC795-spec  |       |       |       |
| 15       | 03VC795-cells |       |       |       |
| 16       | 03VC833-spec  |       |       |       |
| 17       | 03VC833-cells |       |       |       |
| 18       | 03VC806-spec  |       |       |       |
| 19       | 03VC806-cells |       |       |       |
| 20       | RC-1          |       |       |       |
| 21       | APC-hsv1-2    |       |       |       |
| 22       | SPC-hsv-2     |       |       |       |



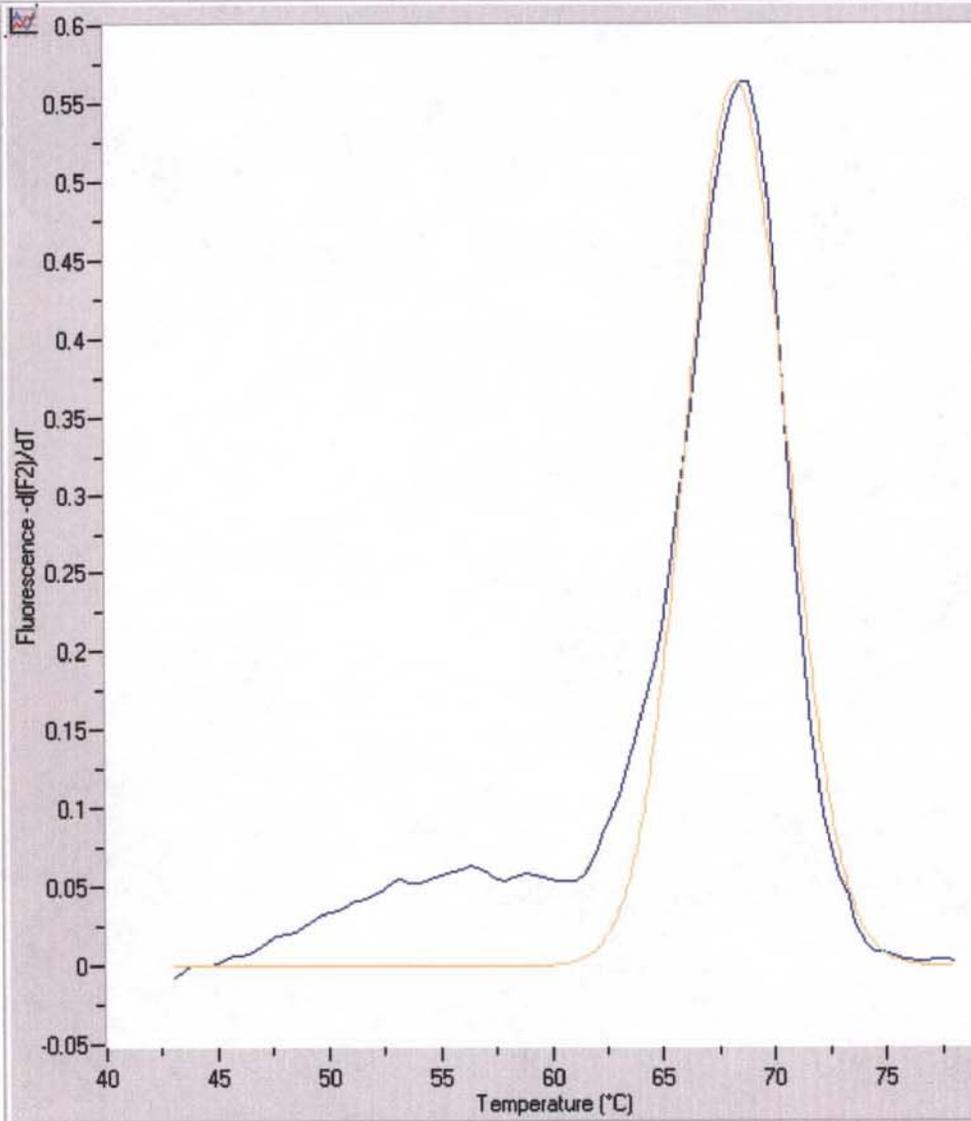
HSV 1 Tm = 54°C

Number of Peaks  
Zero  
One  
Two  
Three

Clear All  
 Weighted Fit

Analysis Notes

| Position | Name          | Tm 1  | Are... | SD 1  |
|----------|---------------|-------|--------|-------|
| 1        | H2O           |       |        |       |
| 2        | NC-1          |       |        |       |
| 3        | 03VC1052      |       |        |       |
| 4        | 03VC1055      |       |        |       |
| 5        | 03VC1057      |       |        |       |
| 6        | 03VC1063      |       |        |       |
| 7        | 03VC1081      | 68.34 | 3.185  | 2.255 |
| 8        | 03VC1083      |       |        |       |
| 9        | 03VC1088      |       |        |       |
| 10       | 03VC1091      |       |        |       |
| 11       | 03VC1097      |       |        |       |
| 12       | 03VC1342      |       |        |       |
| 13       | 03VC1046      |       |        |       |
| 14       | 03VC795-spec  |       |        |       |
| 15       | 03VC795-cells |       |        |       |
| 16       | 03VC833-spec  |       |        |       |
| 17       | 03VC833-cells |       |        |       |
| 18       | 03VC806-spec  |       |        |       |
| 19       | 03VC806-cells |       |        |       |
| 20       | RC-1          |       |        |       |
| 21       | APC-hsv1-2    |       |        |       |
| 22       | SPC-hsv-2     |       |        |       |



HSV 2 Tm = 68°C



# LightCycler Melting Peaks Report

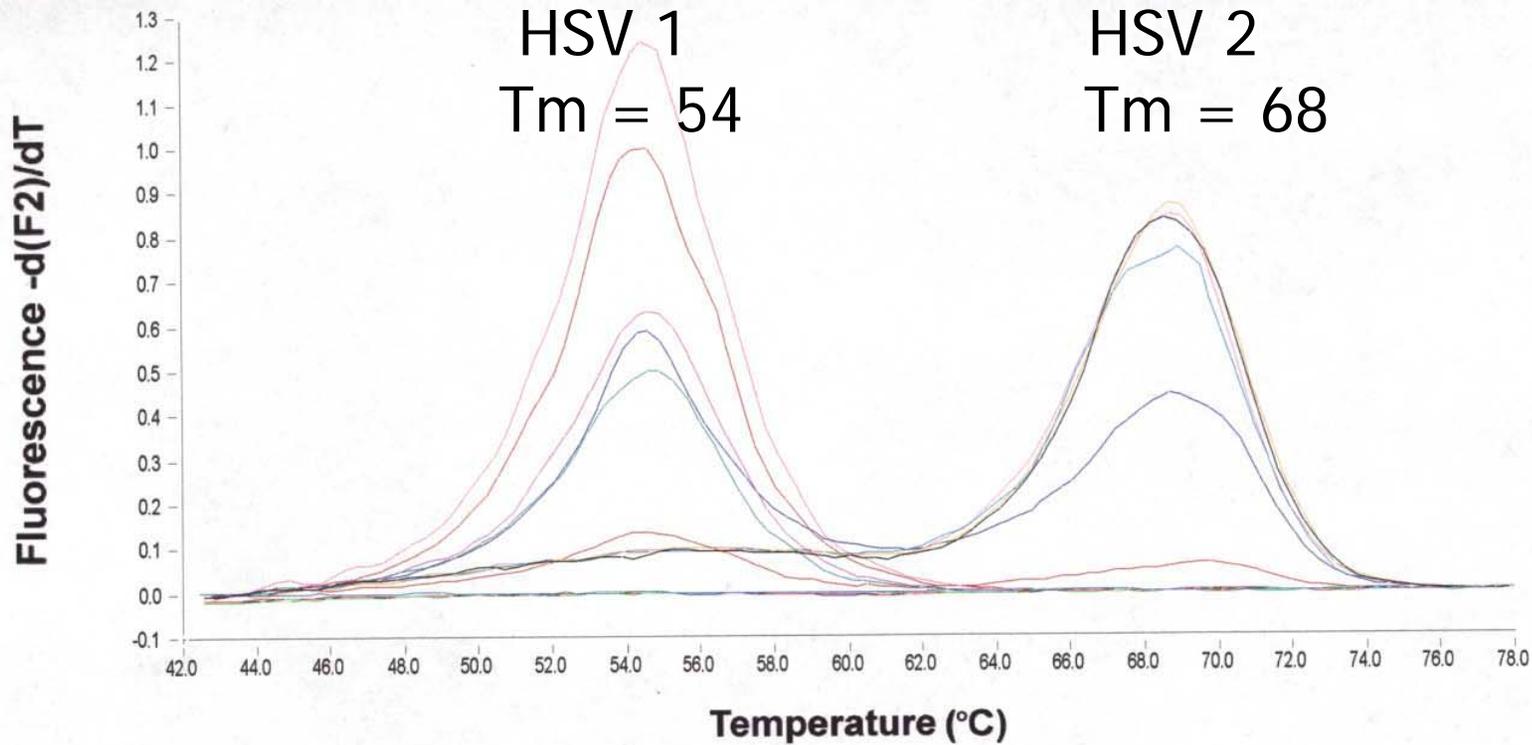
User: rodemank LightCycler ID#: 1323

Run Version: 6.32 Analysis Version: 3.5.28

File: C:\LightCycler3\Users\rodemank\Data\roche-validation-hsv1-2-10-14-03-kmr.ABT Program: PCR-Amplification Run By: rodemank

Run Date: Oct 14, 2003 14:59 Print Date: October 14, 2003

- 1 H2O
- 2 NC-1
- 3 1r
- 4 2r
- 5 3r
- 6 4r
- 7 5r
- 8 6r
- 9 7r
- 10 8r
- 11 9r
- 12 10r
- 13 11r
- 14 12r
- 15 13r
- 16 RC-1
- 17 APC
- 18 SPC-HSV-1



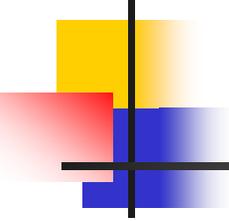
Digital Filter: Enabled

Calculation Method: Polynomial with Background

Lower Background Cursors: 42.46/  
44.48

Upper Background Cursors: 76.83/  
78.85

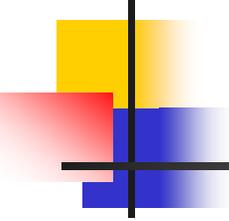
Color Compensation:  
ccc8-28-03.ccc



# 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



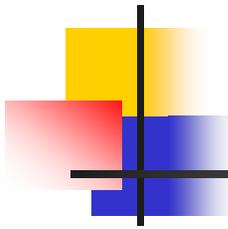
# How to Resolve Discrepancies

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

# Summary of Results

|                  | Neg | PCR<br>HSV1 | PCR<br>HSV2 | Total |
|------------------|-----|-------------|-------------|-------|
| Culture<br>Neg   | 9   | 0           | 1           | 10    |
| Culture<br>HSV 1 | 0   | 8           | 1           | 9     |
| Culture<br>HSV2  | 0   | 0           | 11          | 11    |

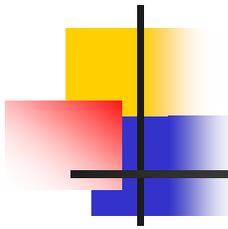


# Ct and Tm Data

|       | Culture | #  | Ct   | Tm   |
|-------|---------|----|------|------|
| HSV-1 | 9       | 8  | 26.5 | 54°C |
| HSV-2 | 11      | 11 | 25.8 | 68°C |

# Summary of Performance Characteristics

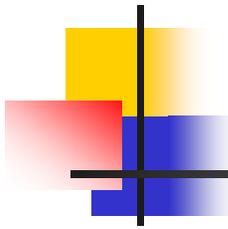
|                        | Actual Results               | Lab Goal           |
|------------------------|------------------------------|--------------------|
| Accuracy               | 95.2%                        | 95%                |
| Precision              | 100%                         | 95%  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



# Evaluation of Non-FDA Approved HSV Assay

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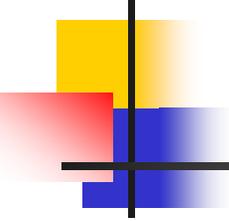
- Detection of HSV 1 & 2 by rapid-cycle real-time PCR using FRET
- Amplifications performed on Roche LightCycler
- HSV 1 and HSV 2 differentiated by melting temperature
  - HSV 1 = 54°C
  - HSV 2 = 68°C



# Summary of Evaluation Study

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- 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



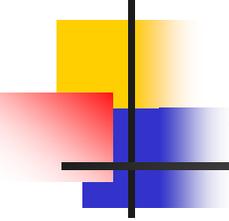
# Final Step - Implementation

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- 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

# References

- The CLIA regulations, [www.phppo.cdc.gov/clia/default.asp](http://www.phppo.cdc.gov/clia/default.asp)
- CLIA Interpretive Guidelines Subpart K, <http://www.cms.hhs.gov/clia/appendc.asp>
- 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](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>



# Acknowledgements

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- NLTN
- APHL
- Nancy Anderson
- Karen Mulawski, MA, MT(ASCP)SC
- MDCH Molecular Biology Staff
  - Eric Belk
  - Steve Dietrich
  - Laura Guild
  - Virginia Leykam
  - Sonia Lugo
  - Teresa Miller
  - Laura Mosher
  - Kevin Rodeman