Verification of PCR

Anyalyte Specific Reagent (ASR) Products

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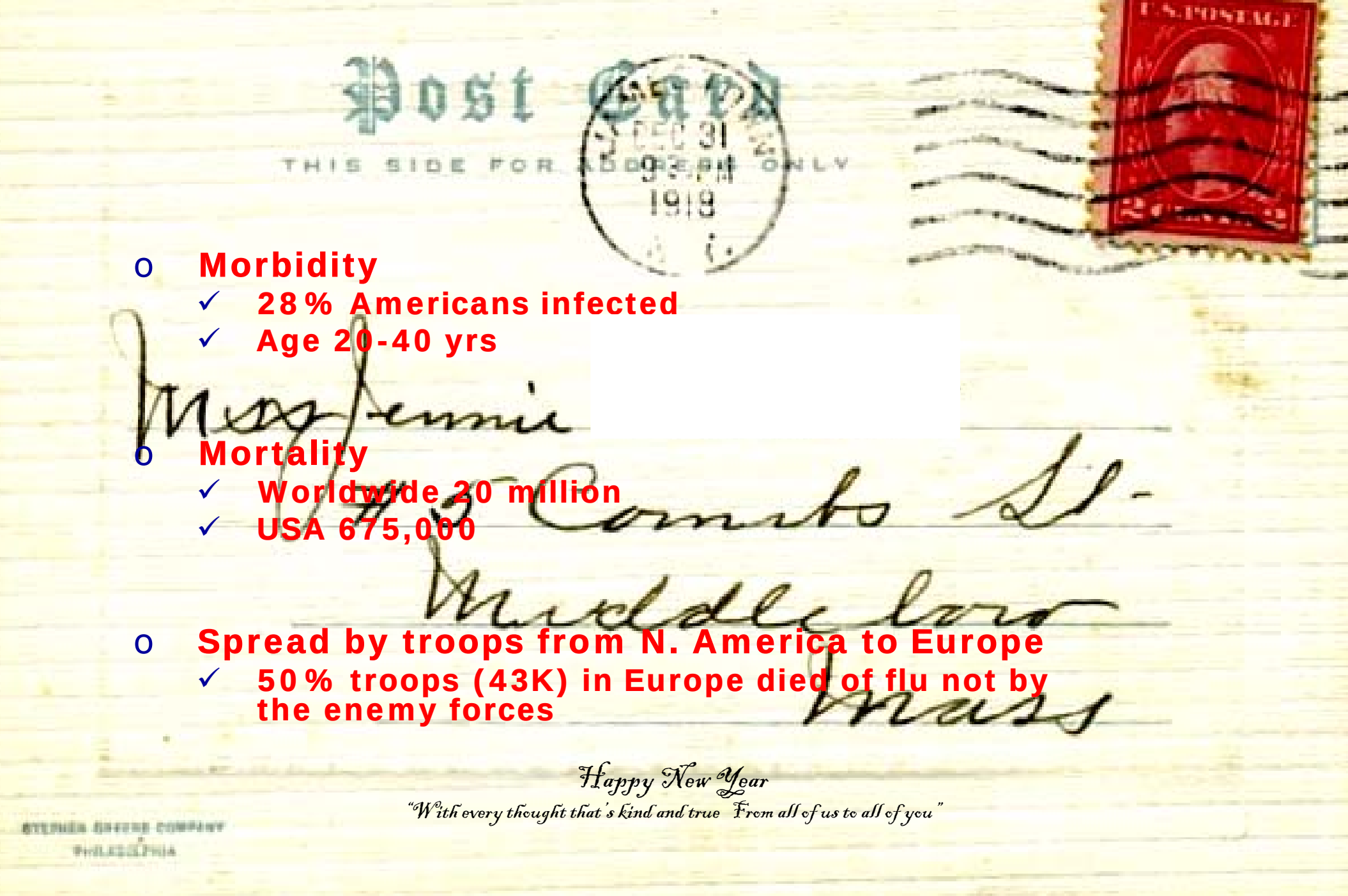
1918 Flu Pandemic

- "Spanish Flu" or "La Grippe" of 1918 -1919
  - Swine-like
- Influenza type A (H1N1)
  - Genes from an avian reservoir

Molly Billings, June, 1997



- **Morbidity**
  - ✓ **28% Americans infected**
  - ✓ **Age 20-40 yrs**
- **Mortality**
  - ✓ **Worldwide 20 million**
  - ✓ **USA 675,000**
- **Spread by troops from N. America to Europe**
  - ✓ **50% troops (43K) in Europe died of flu not by the enemy forces**

Happy New Year

"With every thought that's kind and true From all of us to all of you"

FDA and Analyte Specific Reagents

- ASR's are the building blocks ("Active Ingredients") of in-house developed tests produced and sold by manufacturers
  - ASR Definition (21 CFR 864.4020) "Antibodies, both polyclonal and monoclonal, specific receptor proteins, ligands, nucleic acid sequences, and similar reagents which, through specific binding or chemical reaction with substances in a specimen, are intended to use in a diagnostic application for identification and quantification of an individual chemical substance or ligand in biological specimens."
- Regulation of in-house developed ("home-brew") tests *Federal Register* of November 21, 1997 (62 FR 62260) – Effective November 23, 1998
  - Incremental regulation of both manufacturer and clinical laboratory
    - Clarify FDA oversight for in-house tests in relation to oversight by CMS under CLIA88
    - Provide incremental controls to assure the quality of reagents made over time to the FDA's quality system regulations
    - Clinical laboratory develops, establishes and maintains test performance
    - Manufacturer provides appropriate labeling

FDA and Analyte Specific Reagents

- Manufacturer
  - Register and list with FDA [21 CFR Part 807]
  - Follow quality system regulations [21 CFR Part 820]
  - Label class I exempt as "Analyte Specific Reagent. Analytical and performance characteristics are not established."[21 CFR 809.10(e)(1)(x)]
  - Only sell to high complexity CLIA laboratories
- Laboratory
  - Certified as high complexity under CLIA
  - Establish and maintain performance under CLIA
  - Label the class I ASR test result as "This test was developed and its performance characteristics determined by [Laboratory Name]. It has not been cleared or approved by the U. S. Food and Drug Administration." [21 CFR 809.30(e)]

Contact: Steven Gutman, M.D. sig@cdrh.fda.gov

CLIA Regulations

- CLIA '88
- Feb 28, 1992 CLIA regulations
  - Patient test management
  - Quality control
  - Proficiency testing
  - Personnel
  - Quality assurance
- Additional changes on Dec 6, 1994, May 12, 1997, Oct 14, 1998, Dec 29, 2000
- The 'final final', but really 'not final' rule on Jan 24, 2003 changes concerned the following
  - Technical standards update (re-designated quality system)
  - Revised personnel qualification requirements for high complexity laboratory director

CLIA Law

CLIA applies to all facilities that perform:

"examination of materials derived from the human body for the purpose of providing information for the diagnosis, prevention, or treatment of any disease or impairment of, or the assessment of the health of, human beings…."

CLIA is applicable to all clinical testing.

Verification and Validation*

- Verification
  - (ISO 9000) Evidence that specific requirements have been met
  - One-time process to confirm test performance before implementation for patient testing
- Validation
  - Evidence to support a specific intended use
    - (WHO-BS95.1793) "the action (or process) of proving that a procedure, process, system, equipment, or method used works as expected and achieves the intended result"
  - Validation components
    - Quality control
    - Proficiency testing
    - Employee competency
    - Instrument calibration
    - Clinical correlation

*MM3-A2 Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline – Second Edition. CLSI. 2006.

Method Validation and Quality Assessment

- Method validation
- Method quality assessment ("assurance")
- What is necessary for method validation?
- What is required for method validation?
  - CLIA '88 and the final, but never final documents
  - College of American Pathologists (CAP)
  - Clinical and Laboratory Standards Institute (CLSI)
  - Specific state requirements with exempt status (e.g. NY)
- When do you need to revalidate a test?
- What is the action needed if validation or revalidation fails?

Requirements for Verifying or Establishing Performance Specifications

- Applies to each nonwaived test system introduced on or after April 24, 2003
- Requirements pertain to
  - A test system introduced for the first time
    - New analyte
    - Analyte previously measured/detected on an alternate system
  - An analyte added to a test system
  - A modification to a test system

Quality Management and Quality Control (QM/QC) [10/6/2005]

Assay Validation (MOL.30785 – MOL.32050)

- Refers to "verification"
  - Laboratory-developed (in-house) assays
  - Laboratory-modified FDA-cleared assays
- Performance characteristics
  - Sensitivity, specificity, precision, linearity
    - Analytical
    - Clinical (Diagnostic)
      - Relative to the "Gold standard"
      - Relative to the clinical diagnosis or inter-laboratory testing
  - Genotype representation
  - Specimen representation

Challenges in Molecular Test Verification

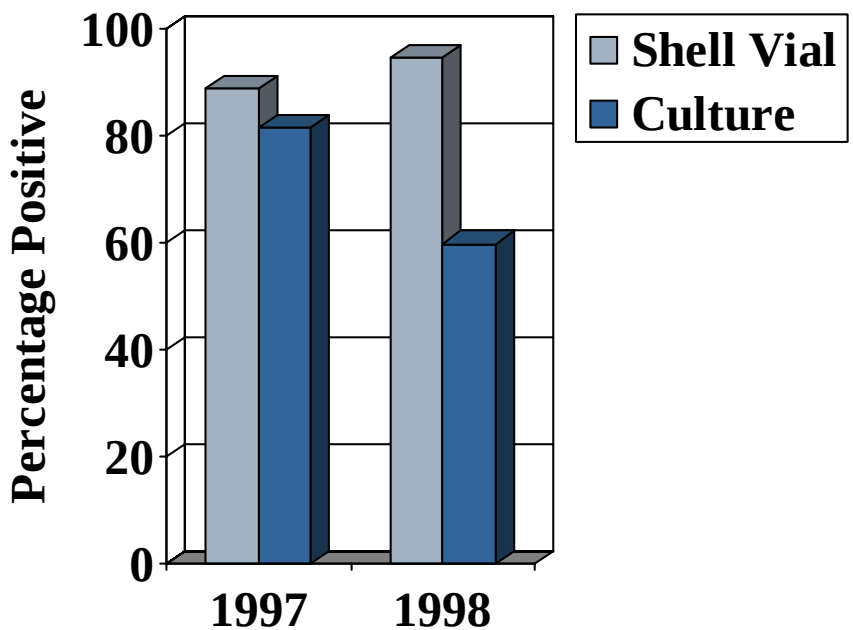
- Molecular testing (e.g. PCR)
  - Increased sensitivity
  - Increased specificity
- Challenge the "Gold Standard" (e.g. culture)
  - Predicate device?
    - Prove substantial equivalence to a legally marketed device [predicate device, usually recent 510(k)]
- Specimens (samples) used in testing
  - Patient, PT, "seeded" specimens ("Matrix")

Influenza Virus - Laboratory Testing

Laboratory Test	Specimen Type	Sensitivity	Time to Result	Comments
Direct fluorescent antibody (DFA)*	Nasal or throat washing, throat swab, bronchial wash, sputum or nasopharyngeal	44.4 % **	1-2 hrs	Specificity 98.8%**
Shell vial Culture		56 - 100%	1-2 days	-
Direct viral antigen (EIA)		50 - 80%	4 - 20 hrs	-
Standard cell culture*		100% (“Gold Standard”)	2-14 days (Virus isolation 2-6 day range and □≡3days)**	Sensitivity could be influenced by therapy, specimen collection time and specimen handling.
Influenza antibody (serology using complement fixation [CF], haemagglutination inhibition [HAI], neutralization test [NT] or enzyme immunoassay [EIA])		94% (Influenza A by CF testing)	1-2 days	Acute and convalescent sera drawn 10-14 days apart. Expect a fourfold increase in titer for IgG levels.

Influenza Virus Type A Detection

ViroMed Laboratories (1997 – 1998)



January 1997*	Shell Vial Pos	Shell Vial Neg
Culture Pos	19 (Wuhan-like)	3 (Wuhan-like)
Culture Neg	4 (Wuhan-like) 1 (No type)	NA

January 1998**	Shell Vial Pos	Shell Vial Neg
Culture Pos	16 (Nanchang-like) 40 (Sidney-like) 22 (Variant?) 36 (No type)	1 (Nanchang-like) 5 (Sydney-like) 5 (No type)
Culture Neg	15 (Sidney-like) 69 (No type)	NA

Performance Characteristics

- Analytical sensitivity measures the smallest quantity that can be reproducibly detected, the detection limit.
  - This value can be defined at the 0.95 confidence interval ± 2 standard deviations.
- Clinical sensitivity is the test "positivity" in a population of affected patients.

- Specificity is the efficiency of a test in ruling out an analyte or disease. Analytical specificity is the measure of a method to identify only the analyte the test is designed to identify.
- Clinical specificity is the percent of negative test results in a population without the specified disease.

- Positive predictive value (PPV)
  - Proportion of patients with *positive* tests who have disease

- Negative predictive value (NPV)
  - Proportion of patients with *negative* tests who *do not* have disease

Primer Sensitivity*

Table 3: Sensitivity of H5N1 primers compared to WHO recommended H5 primers.

Sample Type	Subtype	Limit of detection WHO recommended H5 primers	Limit of detection H5N1 primers described here
Human 3028	H5N1	10^{-1}	10^{-3}
Avian 933	H5N1	10^{-1}	10^{-3}
Avian 949B	H5N1	10^{-3}	10^{-4}

Human 3028: A/Vietnam/3028/2004
Avian 933: A/Chicken/Vietnam/933/2004
Avian 949B: A/Chicken/Vietnam/949B/2004

***Specific detection of H5N1 avian influenza A virus in field specimens by a one-step RT-PCR assay**
Lisa FP Ng, et.al. *BMC Infectious Diseases*2006, **6**:40

Primer Specificity*

Table 1: Human specimens used as controls in one-step RT-PCR H5N1 assay

Pathogen	Early disease symptoms	*n*
Virus:		
Respiratory syncytial virus (RSV) B	"Flu"-like	1
Dengue 1	Fever, "Flu"-like	1
Dengue 2	Fever, "Flu"-like	1
Dengue 3	Fever, "Flu"-like	1
Dengue 4	Fever, "Flu"-like	1
Severe respiratory syndrome virus (SARS)	High fever, dyspnea, malaise	2
Hepatitis B virus (HBV)	"Flu"-like, malaise	6
Bacteria:		
Haemophilus influenzae	Fever, "Flu"-like	1
Legionella pneumopnila	"Flu"-like, pneumonia	1
Klebsiella pneumoniae	"Flu"-like, pneumonia	1
Streptococcus pneumoniae	"Flu"-like, pneumonia	1
Mycoplasma pneumoniae	"Flu"-like, malaise	1
Mycobacterium	Fever, malaise, dyspnea	1

***Specific detection of H5N1 avian influenza A virus in field specimens by a one-step RT-PCR assay**
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Performance Characteristics

- Accuracy is the closeness of the measurement to the true value (reference)
  - (# correct results/# total results) x 100

- Precision is a measure of the extent to which replicate analyses of a homogeneous analyte agree with each other.
  - Precision is synonymous with reproducibility.
  - Precision is applied to quantitative assays and reproducibility to qualitative assays.
  - (# of repeated results in agreement/# total results) x 100

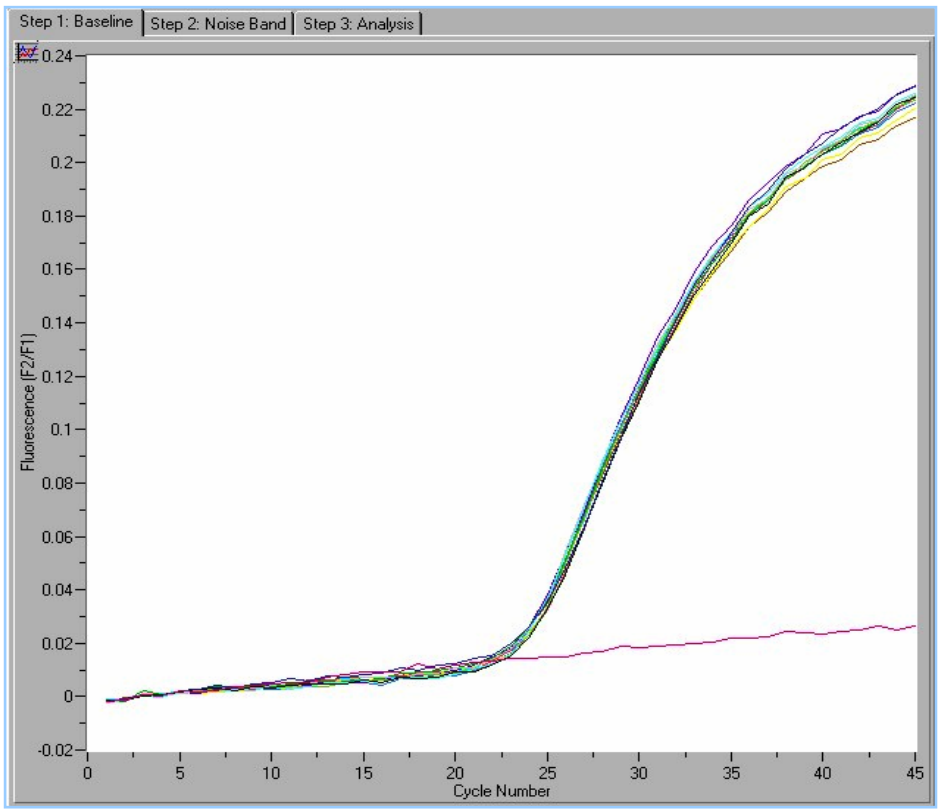
Performance Characteristics

- Test variation and reproducibility
  - Operator
  - Intra-run
  - Inter-run ("run-to-run")
    - Day-to-day
- Specimens
  - Type (site)
  - Inhibition
- Reference range
  - "Normal Range" for quantitative tests
- Demographics (if applicable)

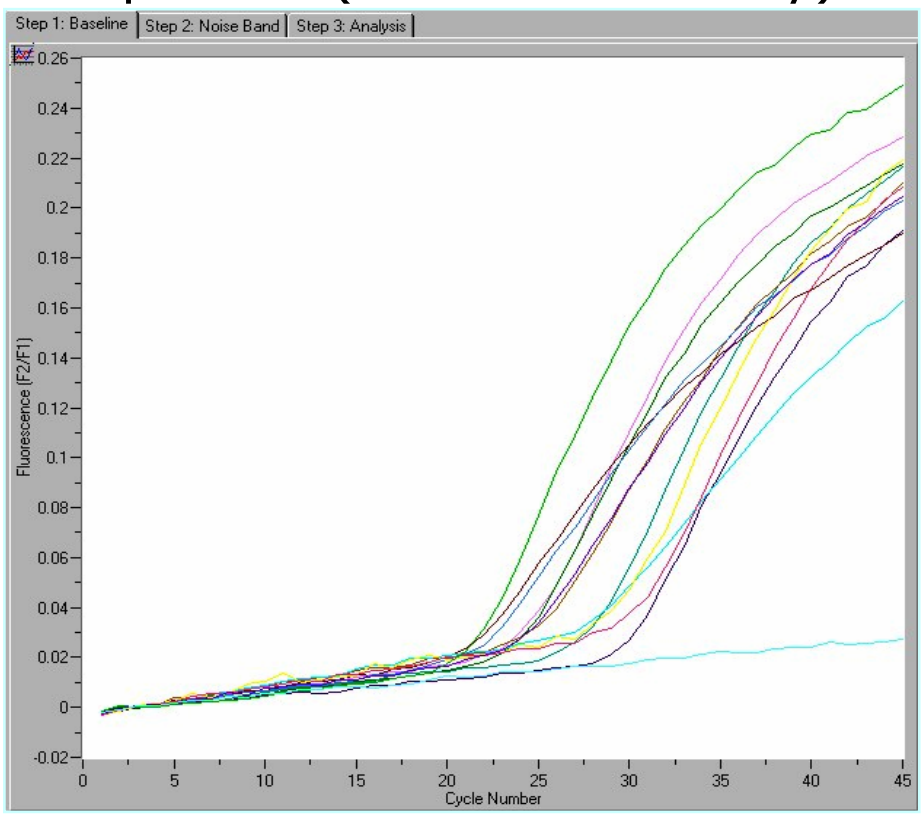
Intra-assay Reproducibility

(Relative efficiency for a known concentration of nucleic acid)

Precise



Not precise (Lack of efficiency)



When should we have larger N*?

- For studies of significant consequence
- If the sample is very diversified
- Minute differences are expected/anticipated
- For longitudinal studies
- If you are to have subgroup analyses
- Attrition of subjects are anticipated
- Test measures are unreliable
- Variables are complex and difficult to control

*** N = Number of measurements in a sample size**

Sample Number and Testing Schedule

- Parallel testing
  - 50-100 specimens
  - 3-7 days
  - Split specimens to reference lab using a comparable method
    - Obtain test performance data sheet
    - Cost of testing
- Clinical specificity
  - At least 20 known isolates or clinical specimens
  - At least 40 known negative

Test Performance

Test Performance Results for Influenza Virus Type A and Type B

Test = PCR **Standard = Shell Vial (SV) Culture**

			PCR	
		401	**Pos**	**Neg**
SV	**Pos**		150	0
	Neg		15	236

Sensitivity=	100.00	[TP/(TP+FN)X100]
Specificity=	94.02	[TN/(FP+TN)X100]
PPV=	90.91	[TP/(TP+FP)X100]
NPV=	100.00	[TN/(TN+FN)X100]
Efficiency=	96.26	[(TP+TN)/(TP+FP+FN+TN)X100]

Test Verification

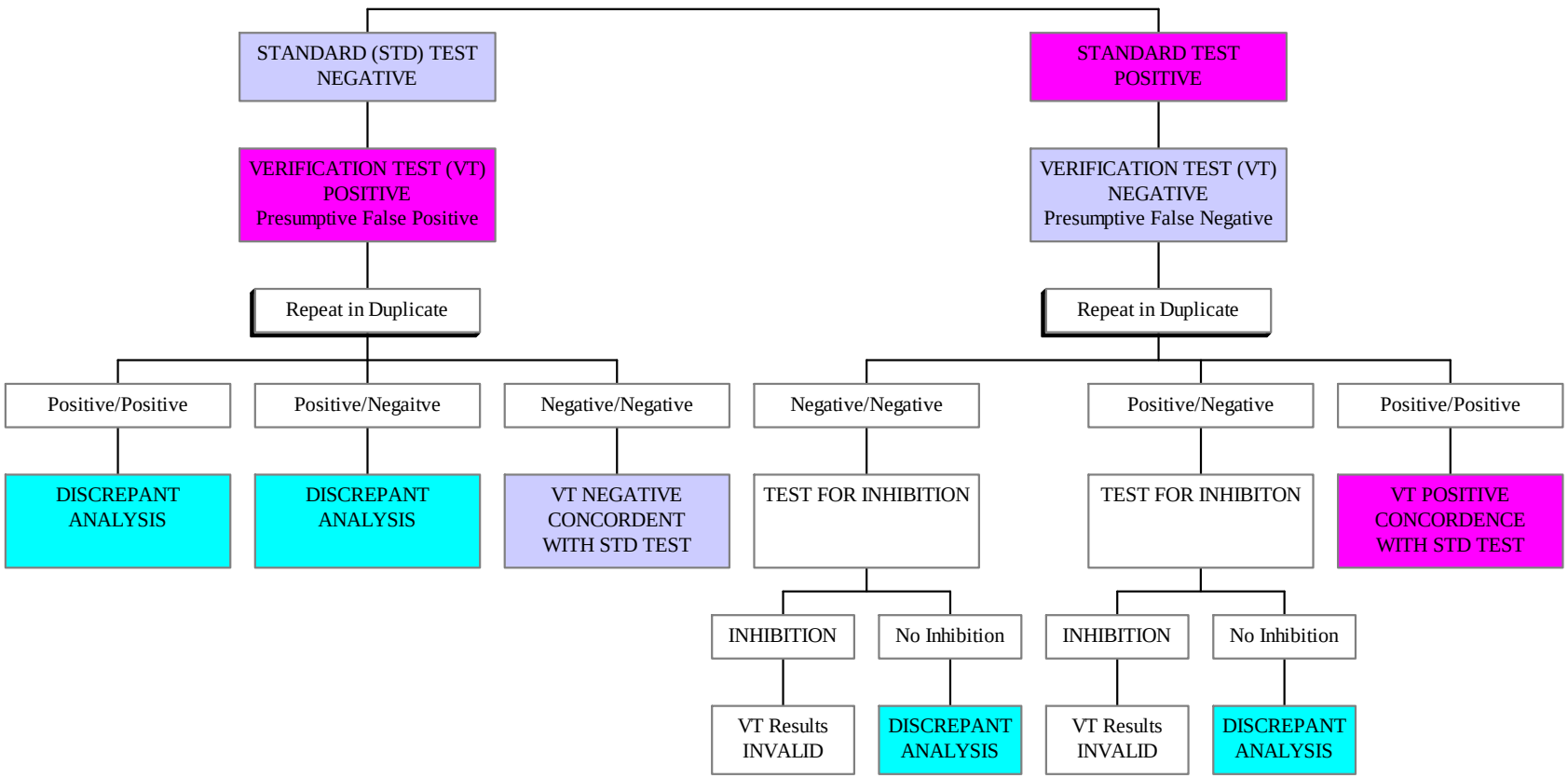
Discrepant Analysis

- Discrepant analysis is performed when the results of the new assay are in disagreement with the standard test results.
  - If the new assay result is positive and the standard assay is negative, the new assay could either be a **false positive** or a true positive not detected by the old assay.
  - If the new assay result is negative and the standard assay is positive the new assay is a **false negative.**

- Discrepant analysis should be performed as indicated in the algorithm.
- Discrepant analysis involves one or more of the following actions
  - Chart review including patient history, drug therapy, and outcome.
  - Additional specific laboratory test results
  - Reference laboratory using a method comparable in test performance (i.e. sensitivity, specificity, accuracy, precision)

Test Verification

Discrepant Testing Algorithm



Discrepant Analysis

Test Performance Results for Influenza Virus Type A and Type B

			PCR*	
		401	**Pos**	**Neg**
Discrepant Analysis	**Pos**		163	0
	Neg		2	236

* Discrepant analysis results after one or more of the following:

1. Additional testing (Alternate target PCR)

2. Chart review

3. Epidemiology investigation

4. Referral to an outside laboratory

Sensitivity=	100.00	[TP/(TP+FN)X100]
Specificity=	99.16	[TN/(FP+TN)X100]
PPV=	98.79	[TP/(TP+FP)X100]
NPV=	100.00	[TN/(TN+FN)X100]
Efficiency=	99.50	[(TP+TN)/(TP+FP+FN+TN)X100]

Criteria for Acceptance

- Test sensitivity and specificity are greater than or equal to 95%
- Accuracy and reproducibility are greater than or equal to 95%
- Other considerations
  - Invalid repeat rate exceeds 5%
  - Reagent issues (e.g. instability)

Performance Characteristics

Test Development Manual

- Nucleic acid sequence characterization
  - Phylogenetic relationship
  - Microbial cross-reactivity
    - Normal flora, pathogens, similar diseases
- Test Development Protocol
- Data and analysis
- SOP
- Test Development Report
- Copies of references
- Test performance technical sheet

Quality Management and Quality Control (QM/QC) [10/6/2005]
Procedure Manual

- Quantitative molecular tests (MOL.30440 – Phase II)
  - Calculation with units defined
    - Dynamic range
    - Controls (Negative, low positive, high positive)
  - Melting curve interpretation (Real-time)
- Analytic interpretation (MOL.30555 – Phase II)
  - Qualitative
    - Band pattern, Tm, numeric c/o
  - Quantitative
    - Run test performance verification
      - Sensitivity, linearity, inhibition

Test Performance Technical Sheet

HSV-1/HSV-2 RealTime PCR

Test Performance

Test Code	456789
Test Description	HSV-1 and HSV-2 RealTime PCR
Test Methodology	Polymerase chain reaction (PCR) and realtime PCR detection probe technology (LightCycler)
PCR Target	Glycoprotein D gene
Analytical Sensitivity	Limit of Detection (5 viral particles/reaction) Reproducibly (16 viral particles/reaction detected 100% of the time)
Analytical Specificity	No cross reactivity has been noted for the following viruses: Cytomegalovirus — Varicella Zoster Virus Adenovirus — Coxsackievirus B Coxsackievirus A — Echovirus Human Herpesvirus type 6(B)
Clinical Sensitivity	100 % (compared to culture) for HSV-1 and HSV-2 detection
Clinical Specificity	100%
Precision	100%
Accuracy	100%
Interfering Substances	Heparin is known to inhibit this PCR test. Other undefined inhibitors may be present in some specimens, but blood, lipids and certain anti-coagulants (EDTA, ACD) are not inhibitory.
Specimens	CSF, vesicular fluid, amniotic fluid, and tissue
References	**Espy, M. J., J. R. Uhl, P. S. Mitchell, J.N. Thorvilson, K. A. Svien, A. D. Wold, and T. F. Smith.** 2000. Diagnosis of Herpes Simplex Virus Infections in the Clinical Laboratory by LightCycler PCR. J. Clin. Microbiol. **38:**795-799. **Mitchell, P. S., M. J., Espy, T. F. Smith, D. R. Toal, P. N. Rys, E. F. Berbari, D. R. Osmon, and D. H. Persing.** 1997. Laboratory diagnosis of central nervous system infections with herpes simplex virus by PCR performed with cerebrospinal fluid specimens. J. Clin. Microbiol. **35:**2873-2877

Final Checklist

- ✓ Review and approval by Laboratory Director
- ✓ ASR disclaimer on all test reports

  "This test was developed and its performance characteristics determined by Diagnostic Laboratory Services. It has not been cleared or approved by the U. S. Food and Drug Administration."
- ✓ Semi-annual test verification
- ✓ Ongoing test validation

Evaluation of a Real-Time PCR Multiplex Test (ProFlu-1™) for the Direct Detection of Influenza Virus Type A, Influenza Virus Type B, and RSV in Clinical Specimens

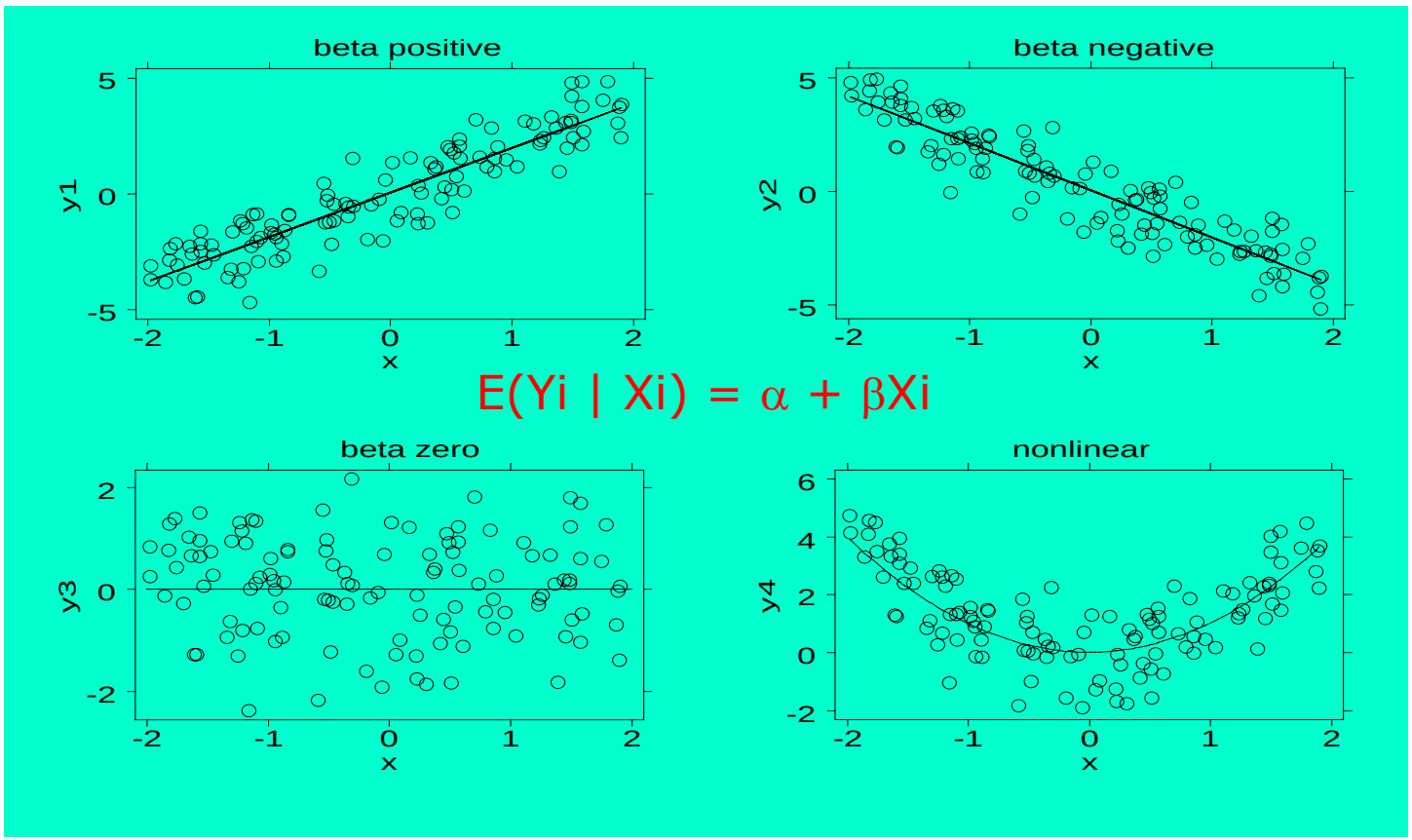
DATA PRESENTED IN SEMINAR

Validation of a Real-Time PCR Test for the Direct Detection and Subtyping of Influenza A Virus in Clinical Specimens

DATA PRESENTED IN SEMINAR

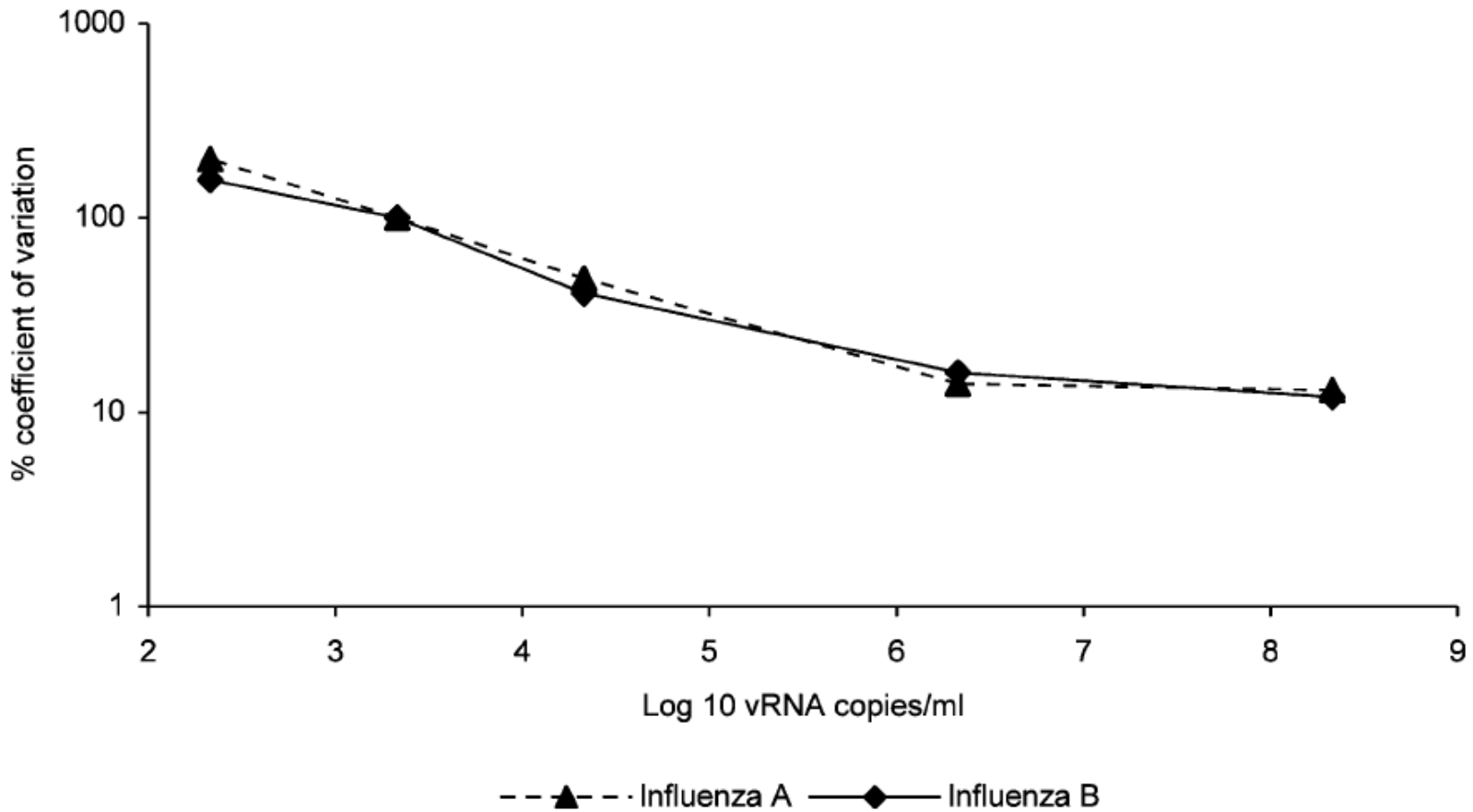
Quantitative Test Considerations

Linear Regression and Beta (Slope) Values



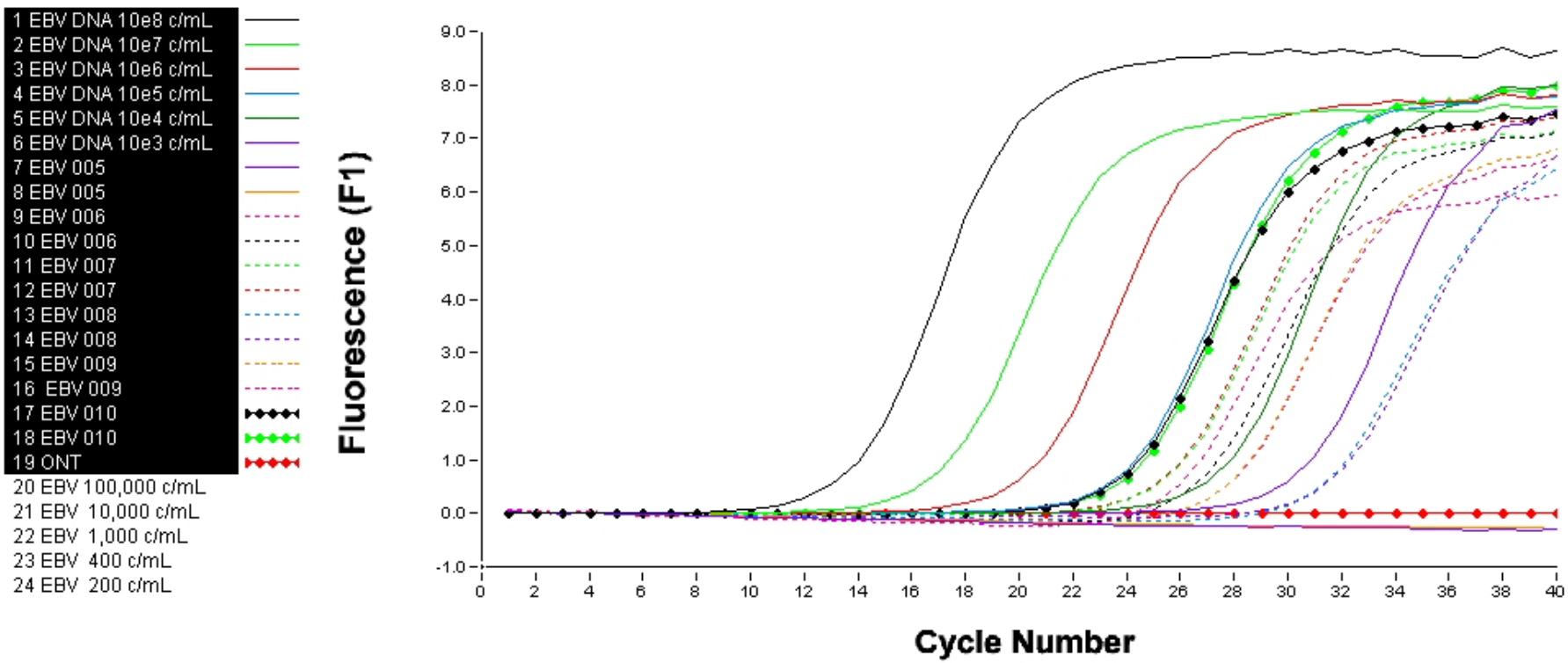
"Strength of association" between two (quantitative) variables

Variation Over the Dynamic Range
for Quantitiative PCR Testing*

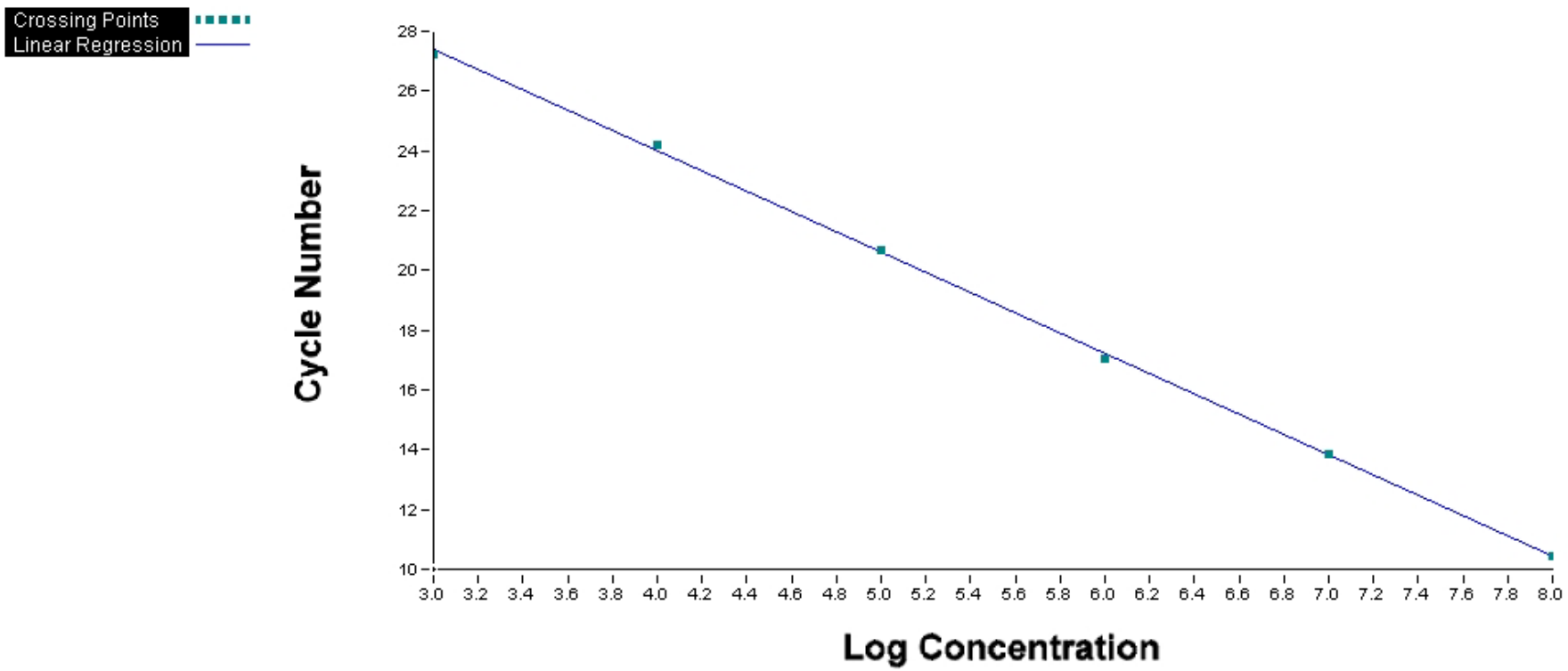


*Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. C.L. Ward, et.al. 2004 J. Clin. Virology 29:179

EBV Crossing Point (Ct)



EBV Standard Curve



Evaluation of the Invader® HCV Genotyping Assay version 1.0 (Clinical Virology Symposium Abstract – 2005)

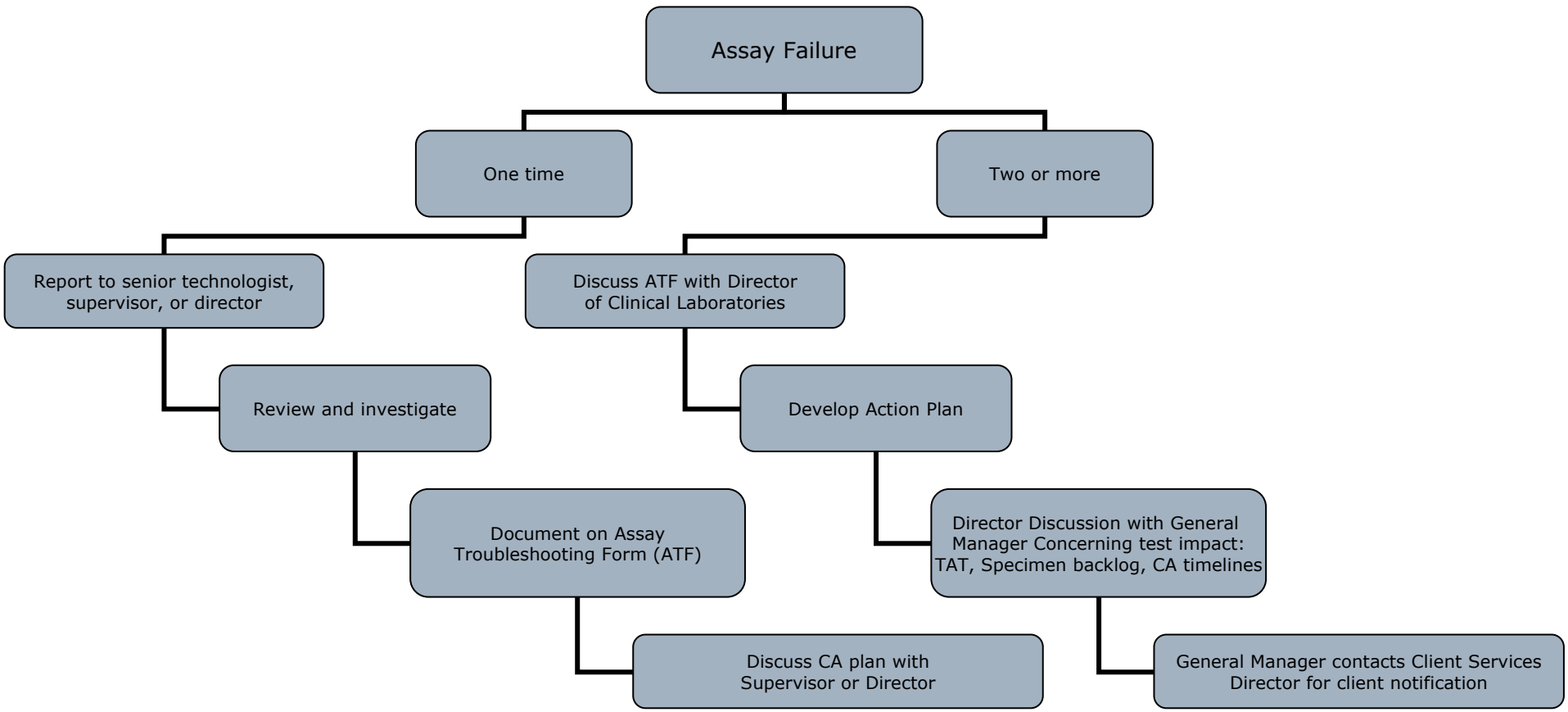
Jeffrey J. Germer, David W. Majewski, Billy Yung, P. Shawn Mitchell, Joseph D. C. Yao
Mayo Clinic, Rochester, Minnesota

HCV RNA concentration (IU/mL)	No. of replicates tested (each assay)	% Replicates successfully genotyped (95% CI)		
		COBAS MONITOR[a]	COBAS AMPLICOR[b]	COBAS TaqMan[c]
5,000	10	100 (69 - 100)	100 (69 - 100)	100 (69 - 100)
1,000	10	100 (69 - 100)	100 (69 - 100)	100 (69 - 100)
500	10	ND	100 (69 - 100)	100 (69 - 100)
100	10	ND	100 (69 - 100)	100 (69 - 100)
50	10	ND	ND	100 (69 - 100)
10	10	ND	ND	100 (69 - 100)
0	10	0 (0 - 31)	0 (0 - 31)	0 (0 - 31)

ND, not done.
[a] Amplification products diluted 1:20.
[b] Amplification products diluted 1:100.
[c] Amplification products diluted 1:500.

Troubleshooting a Verified Test

Assay Failure – Protocol for Assay Troubleshooting



Assay Troubleshooting Form (ATF)

- Assay, date and signatures
- Assay problem description
  - Control failure
  - Excessive background
  - Excessive sample positivity
  - Other problem
- Investigation
- Action plan
- Results of troubleshooting
- Review by laboratory supervisor and clinical director with date of review

Qualitative Test Considerations

- Reagents and Validation

Quality Management and Quality Control (QM/QC) [10/6/2005]
Reagents (MOL.34065 – MOL.34188)

- Verify and document purchased or prepared reagents (MOL.34065 – Phase II)
  - Direct analysis with reference materials
  - Parallel testing (prior or concurrent)
    - Qualitative testing
      - Known positive and negative patient from old lot
    - Quantitative testing
      - Several patient samples at different levels
      - Weakly positive (if reported)
  - Check against routine controls
    - Awareness of "matrix interference"

Quality Management and Quality Control (QM/QC) [10/6/2005]

Reagents (MOL.34065 – MOL.34188)

- Expiration date (MOL.34147 – Phase II)
  - Provided by manufacturer
  - Assigned by laboratory
    - Known stability
    - Frequency of use
    - Storage conditions
    - Risk of contamination
  - Examples of assigned expiration dates
    - BK Virus Real-time PCR (3 months)
    - *B. microtii* Real-time PCR (9 months)

DATA PRESENTED IN SEMINAR

Quality Management and Quality Control (QM/QC) [10/6/2005]

Controls (MOL.34229 – MOL.34557)

- Controls are "surrogates" for patient specimens
- Validation for type of testing
  - Qualitative
    - Positive, negative and low level positive (some cases)
      - When available, appropriate and practical (MOL.34229 – Phase II)
        - Cystic fibrosis (CF) panel
  - Quantitative
    - Two or more levels
      - "Relevant" analytic and clinical decision points (MOL.34270 – Phase II)
- Result verification prior to reporting
  - Unacceptable controls (MOL.34352 – Phase II)
  - Corrective action (MOL.34393 – Phase II)

Quality Management and Quality Control (QM/QC) [10/6/2005]

Controls (MOL.34229 – MOL.34557)

- Controls processed in the same manner and by the same personnel as patient samples (MOL.34434 – Phase II)
  - Control all steps
    - Pre-analytic
      - Specimen preparation
    - Post-analytic
- Monitor trends
  - Quantitative (MOL.34475 – Phase II)
    - Variance (SD, CV)

Test Validation and Trend Analysis

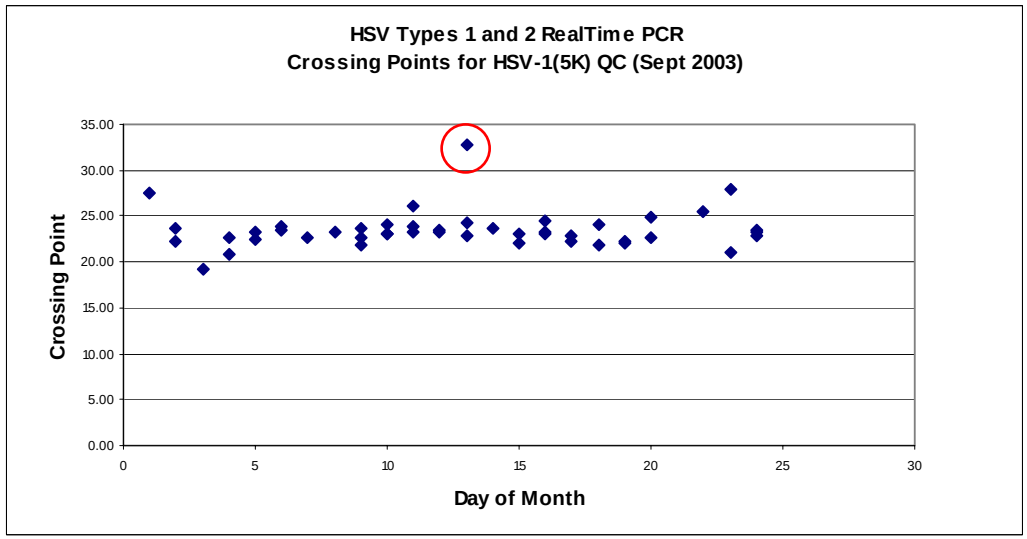
- The documentation that a verified in-house developed test is repeatedly performing according to expectations over time
- Check for biases and changes
  - Crossing point (Ct)
  - Tm
  - New lot compared to old lot of reagents
  - Validation at least semi-annually

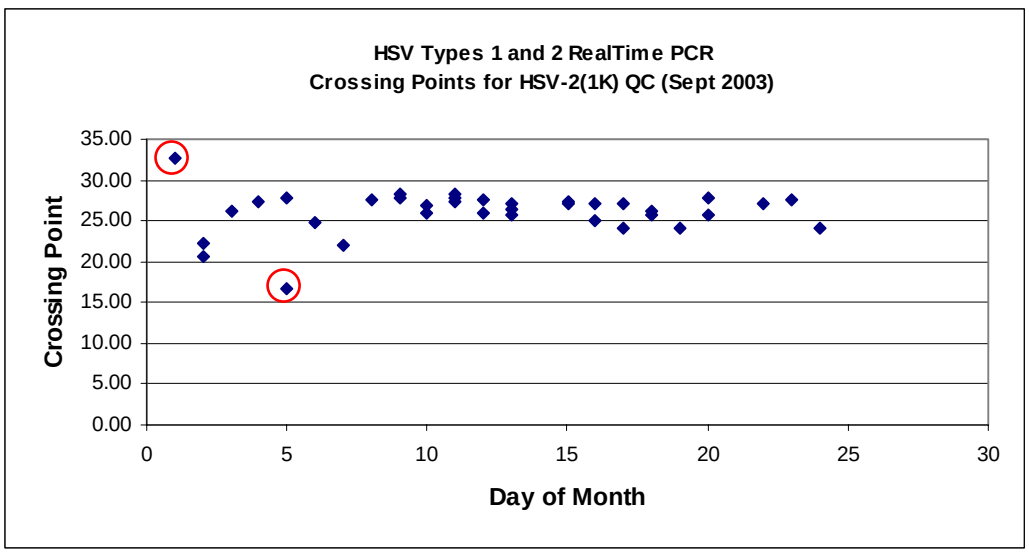
Quality Management and Quality Control (QM/QC) [10/6/2005]

Analysis (MOL.34600 – MOL.35766)

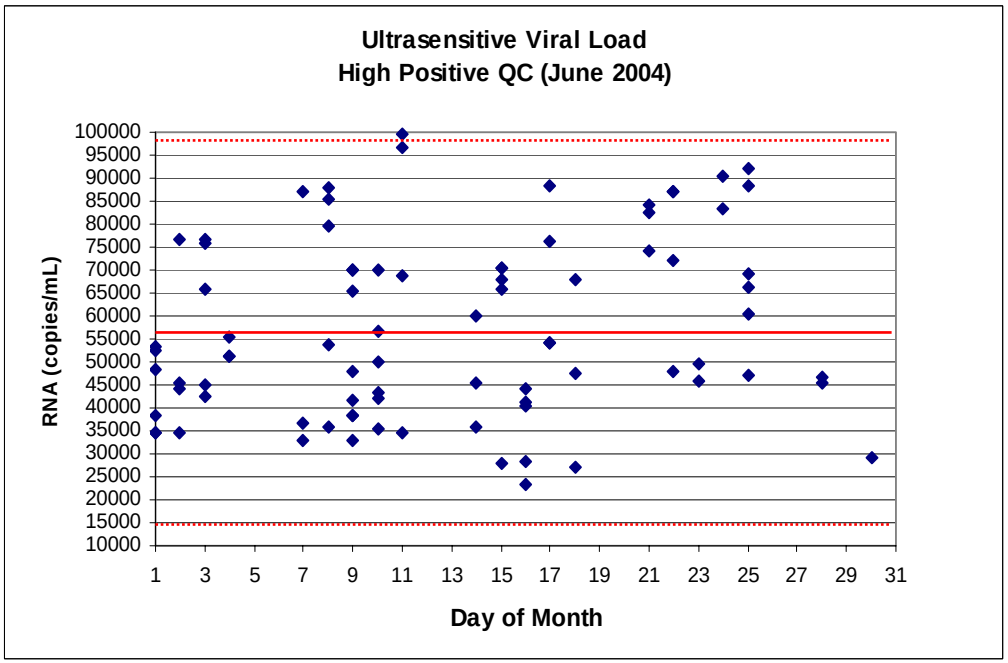
- Real-time PCR
  - Monitor and record
    - T_m result range ≤ +/- 2.5°C
    - Calibrator range (quantitative)
  - Concurrent or pretest of oligo reagents
  - Repeat or investigate IC failures
    - R/O target and IC competition
  - Validate new software against known controls
- Arrays
  - Endogenous positive target
  - Exogenous spiked control
  - Array quality verification

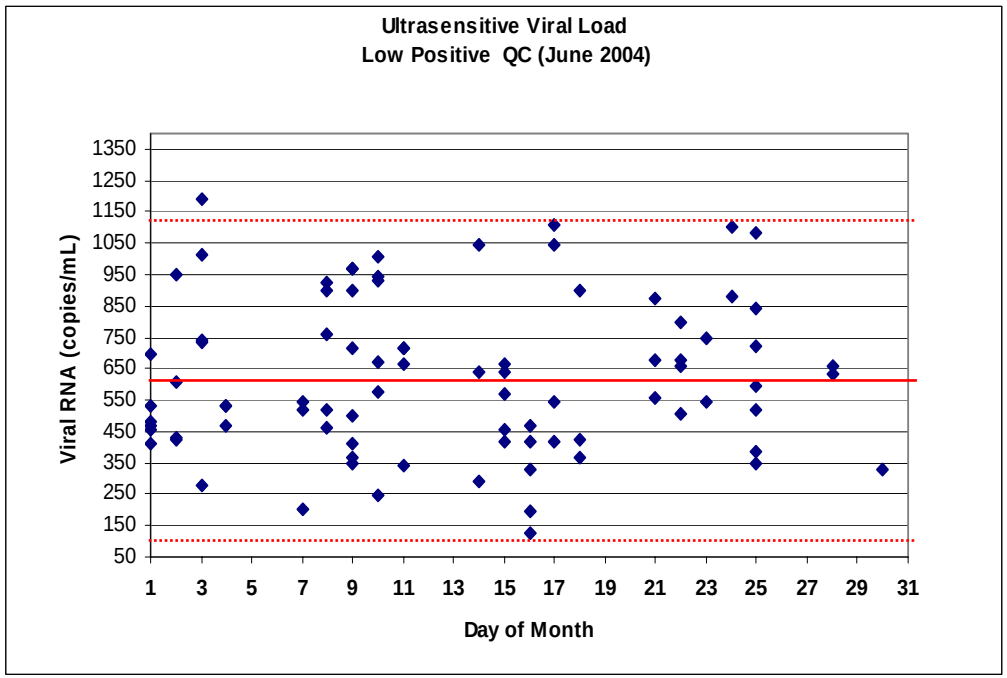
HSV-1/2 RealTime PCR Controls





HIV-1 Monitor PCR Viral Load Controls

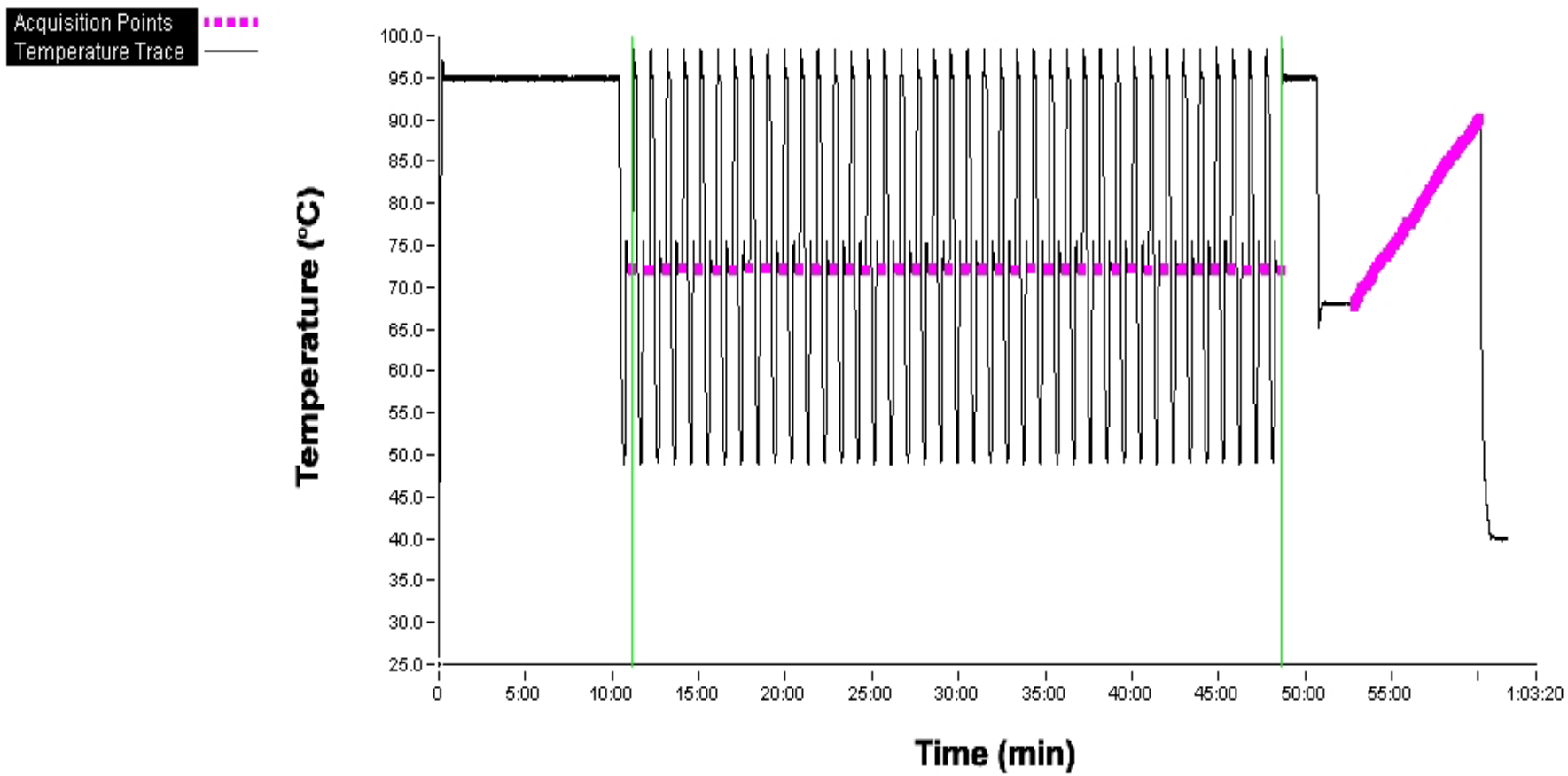




	High Pos	Low Pos
Ave	56667	623
SD	20342	245
Low (-2SD)	15984	133
Hi (+2 SD)	97350	1113

		High Pos		Low Pos	
Date of Testing	**Tech.**	**Ave**	**SD**	**Ave**	**SD**
June 1-4	BK	**51443**	**14076**	**608**	**239**
June 7-9, 21-30	LJ	**60999**	**22380**	**653**	**227**
June 10.	AF	**49592**	**12241**	**729**	**291**
June 11-18	RW	**55675**	**21968**	**563**	**264**

Real-time Amplification Profile



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