Technical Issues with CMV Viral Load Testing and Standards

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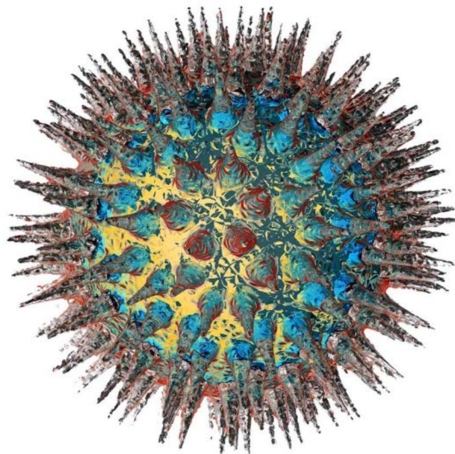


Viral Load Testing- Common Methods

1. DNA Extraction

2. Real-Time PCR Amplification and Detection

Extraction

Extraction (DNA Purification)

* Variety of Methods
  1. Lysis of Cell Membranes
  2. Removal of Proteins
     * Chiatropic agent and a protease enzyme
  3. Binding of DNA to Solid Support
     * Spin Columns with Silica coated on to a membrane
     * Magnetic Beads coated with Silica – Automated Methods with instrumentation (6-96 samples)
  4. Washing and Elution

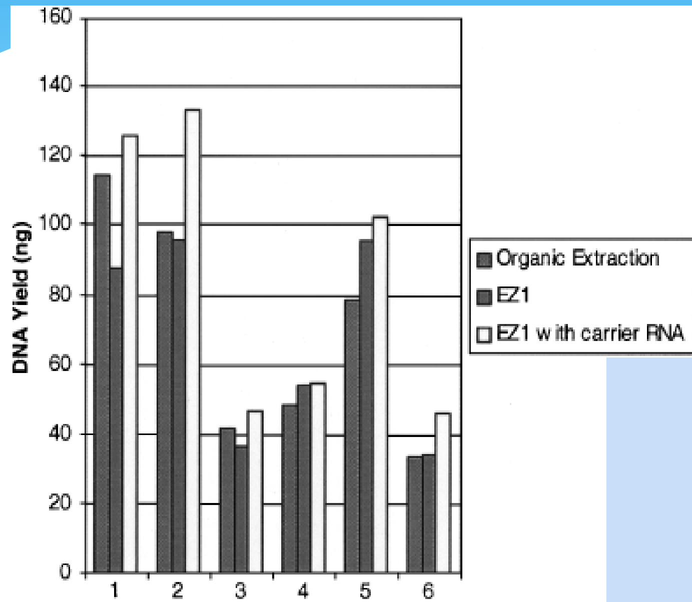
Extraction Evaluation?

- Yield?
- Cross-Contamination?
- Linearity?

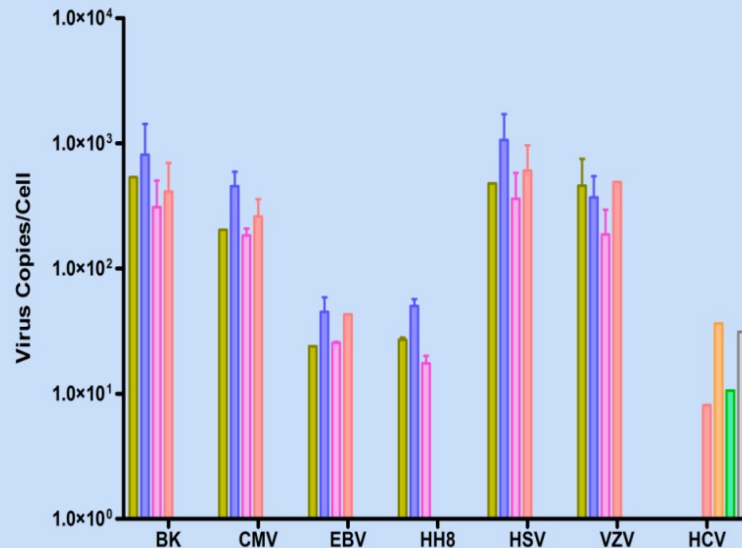
  - In Virology testing usually can only be assessed by PCR measurements
  - Some methods combine both Extraction and PCR so can only assess at the end of both methods.

- For large DNA viruses not a major variable in testing if utilizing commercial extraction equipment/reagents

Measurement of Actual Viral Yield



Kishore, et al. J. Forensic Sci. 51:1156, 2006



Poster, Atienza, A. et al. CVS 2005

Extraction – Sample Type

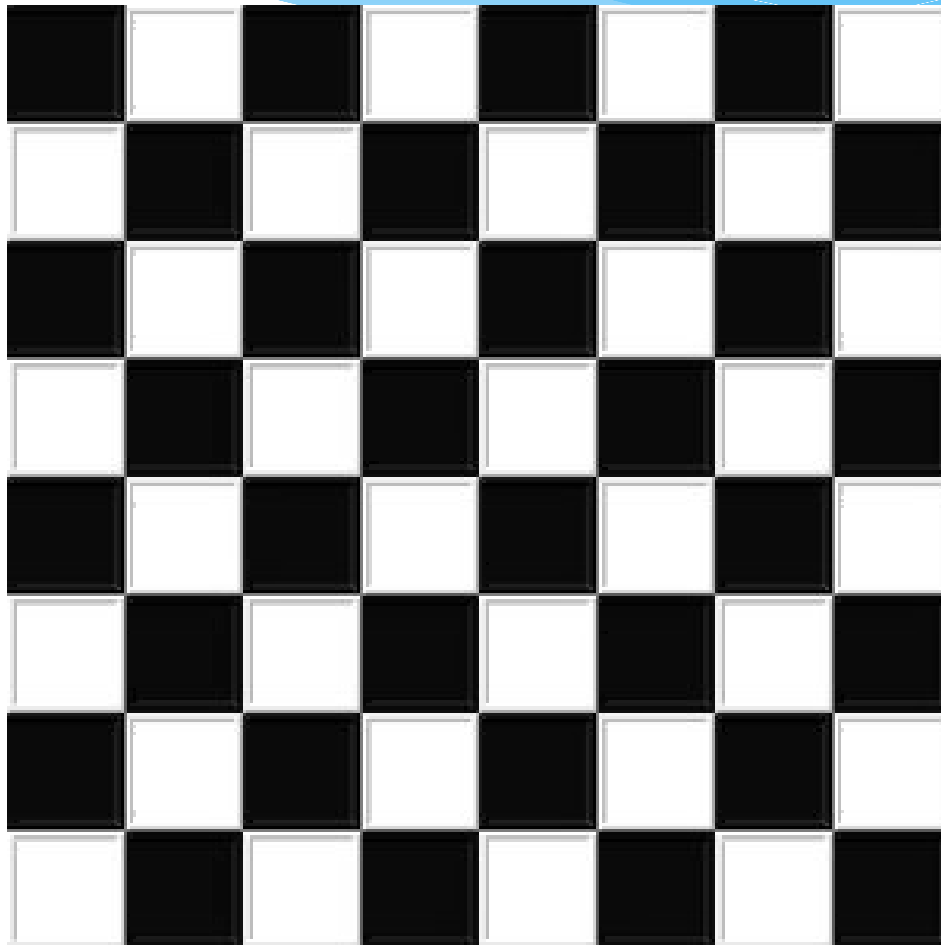
* Plasma – (EDTA)
* Whole Blood
* CSF
* Dried Blood Spots
* Urine

“Checkerboard” Extractions

(Instrument Function)

High Pos

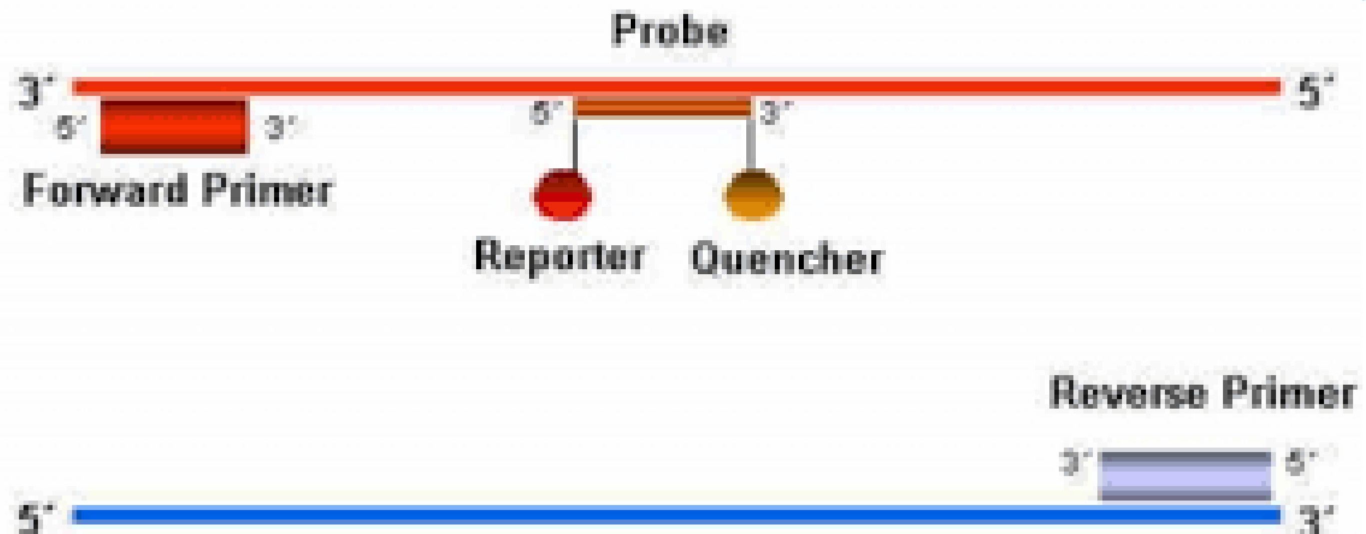
Negative



Technical Aspects - PCR Method

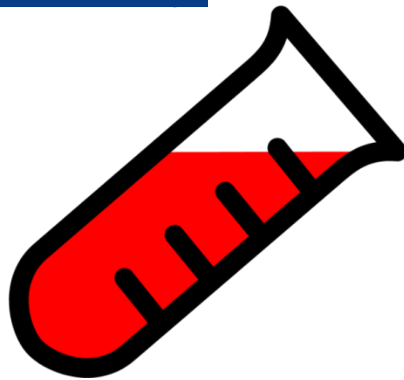
Tools Needed ?

Real-Time PCR



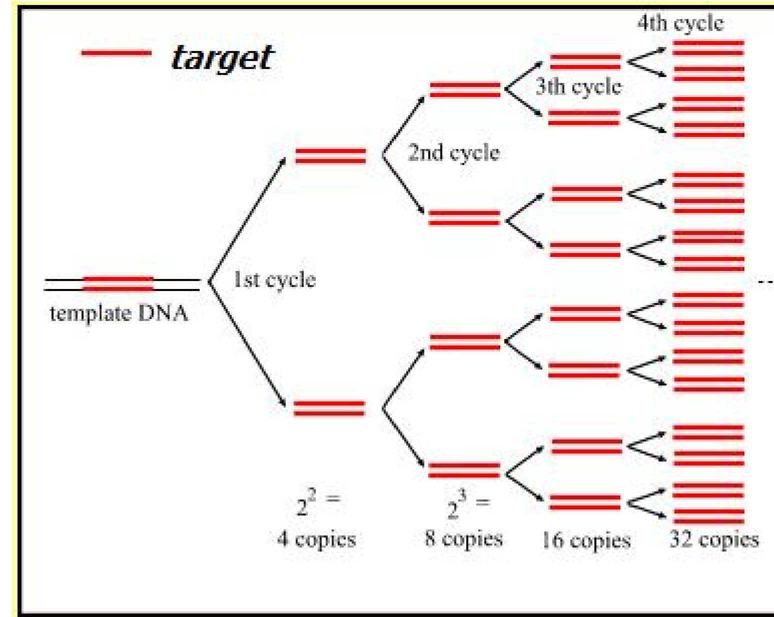
PCR Reaction

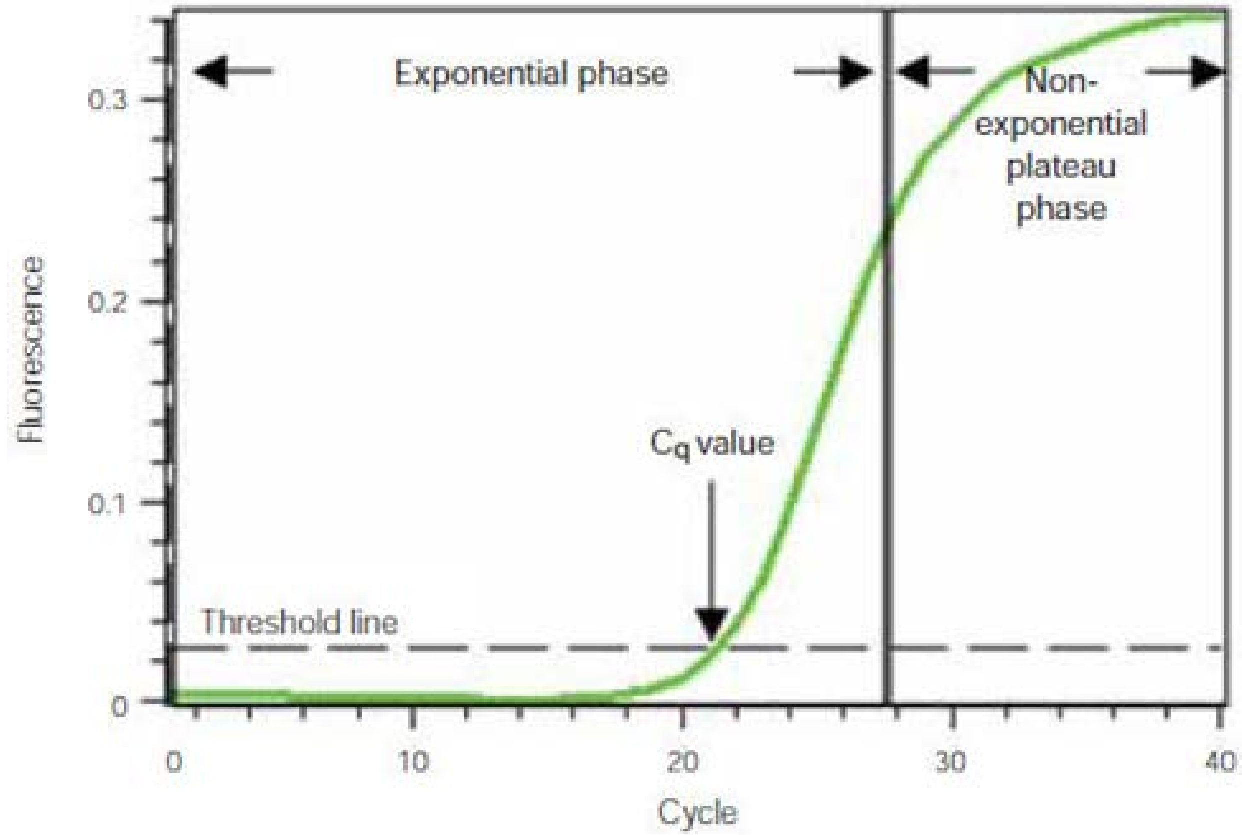
* **Chemistry**



* DNA Polymerase
* Buffers and Cations
* Primers and Probes
* dNTPs
* “Enhancing Agents”

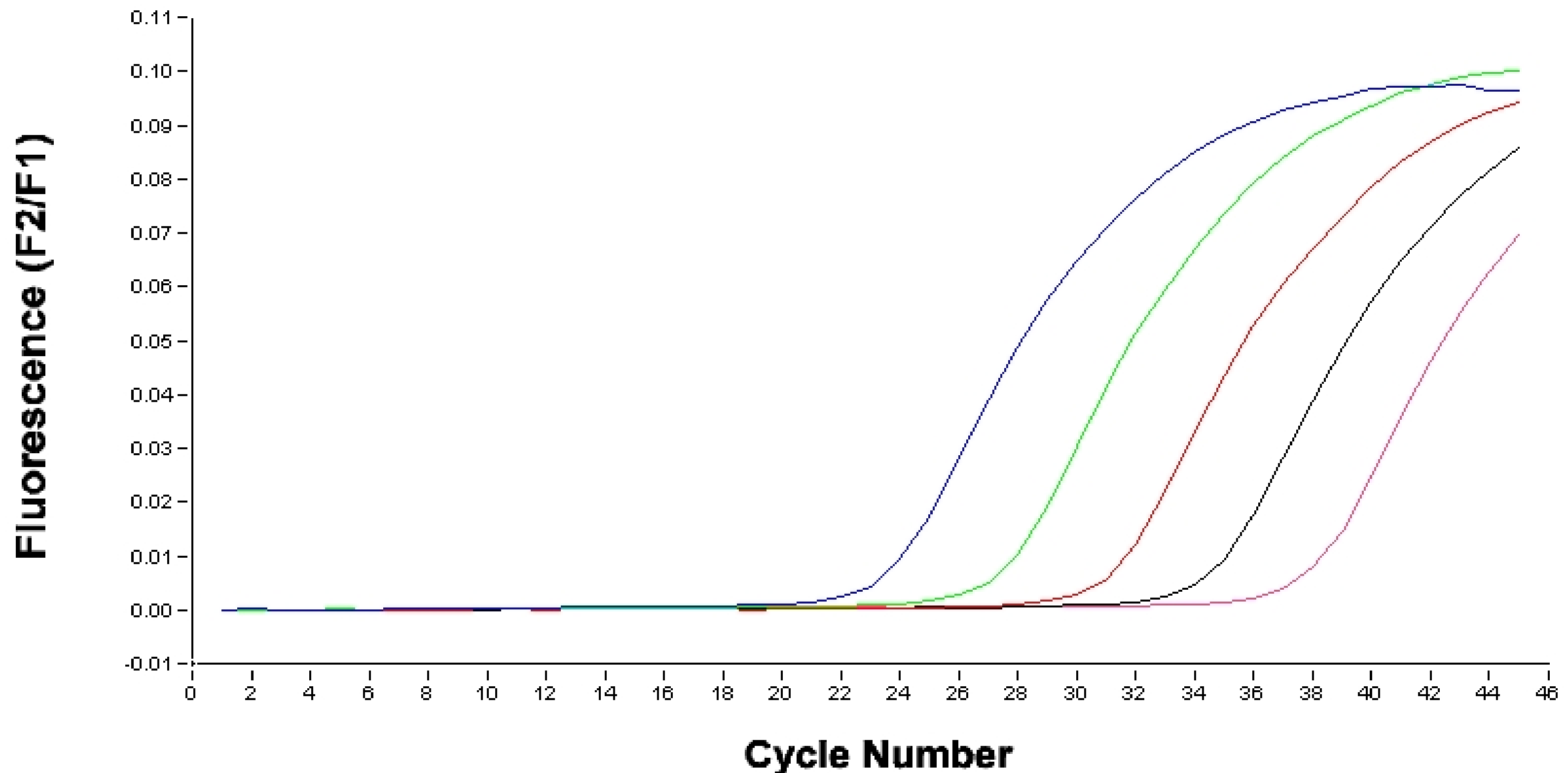
* **Multiple Replication Cycles**




Exponential phase
Non-
exponential
plateau
phase
Fluorescence
0.3
0.2
0.1
0
Cq value
Threshold line
0
10
20
30
40
Cycle

Maximize the Assay Efficiency

- Variety of master mixes available
  - Adjust mix components, conditions, add "enhancers", etc.



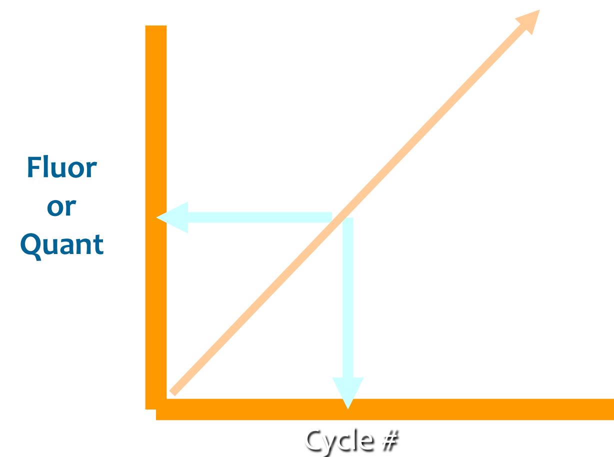
Slope Calculation

Std Curve (Serial dilutions)

Do a Linear Regression – Ct vs Quantity
Calculate the Slope of the Line
-- most Real-Time instruments do this for you.

Efficiency Calculations

* Units – Perfect Efficiency
  * Start with 1 copy
  * End of 1st cycle copy = 2
  * End of 2nd cycle copy = 4
  * End of 3rd cycle copy = 8
  * End of 4th cycle copy = 16

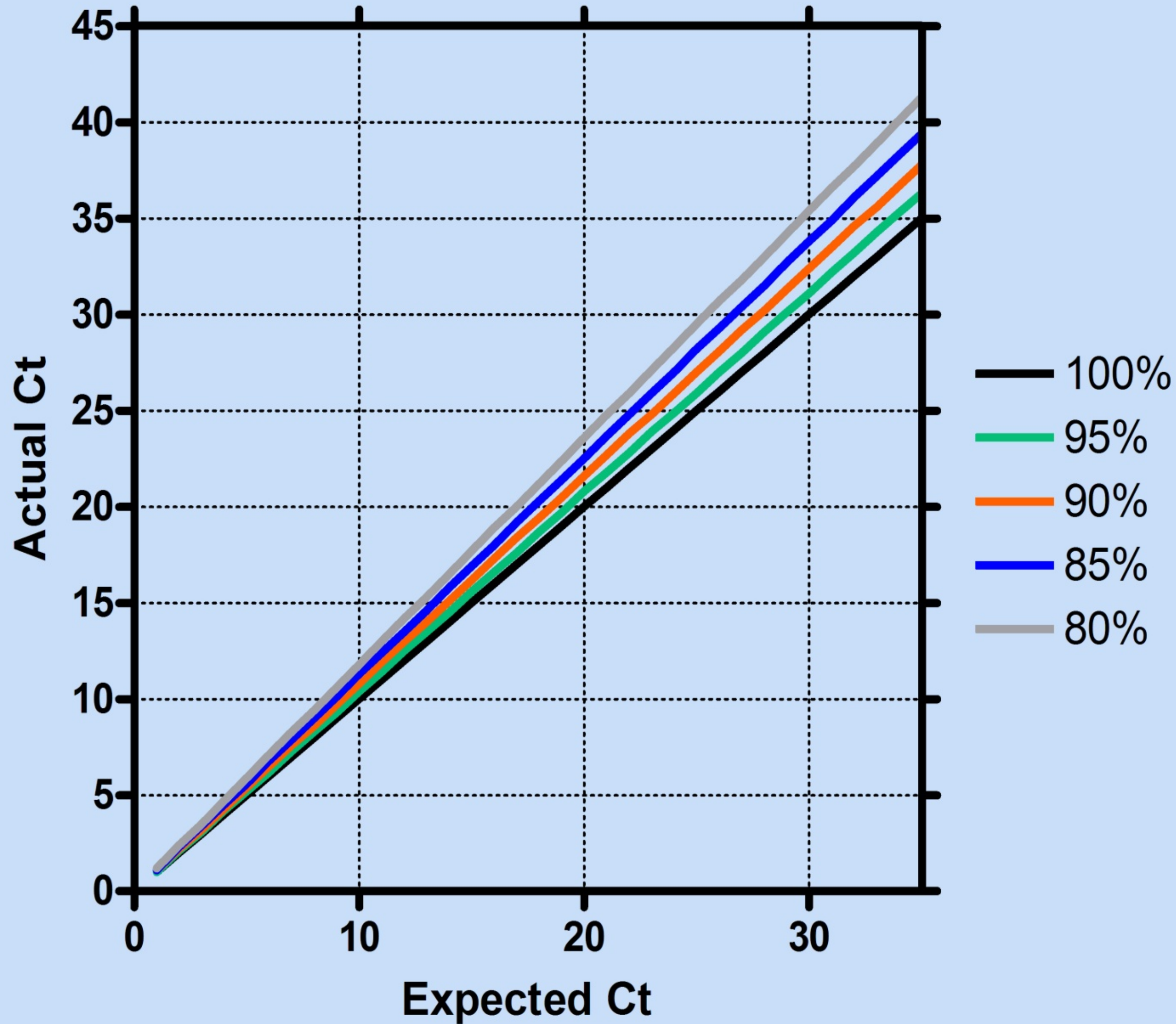


10 fold increase = 3.32 Cycles

Statistical Measures of Assay Efficiency

- Slope of line
  - Perfect is 3.32 Range = 3.12 – 3.52
- Efficiency
  - Perfect is 100% Range = 90-110%
    - $E = -1 + 10^{(-1/slope)}$
- Exponential Amplification
  - Perfect is 2.0 Range = 1.81-2.02
    - $E = 10^{(-1/slope)}$

www.Gene-Quantification.info



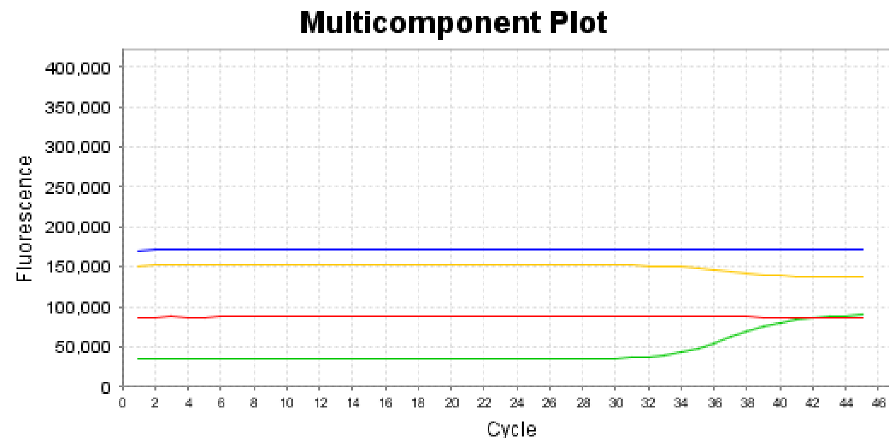
Authors – a 5% difference in efficiency results in a 2 fold difference in product quantity after 26 cycles.

Data shows at 35 cycles – 6.3 cycles or about 2 logs difference between 100% and 80% efficiency

Arikawa, E and Yang, J. SuperArray Bioscience Corp, Fredrick, MD

2nd Check on PCR Assay Performance

* "Internal Control"
  * Another unrelated piece of DNA added to the amplification mix
    * Often added into the sample before extraction
    * Material goes through the entire process
    * Master mix contains primer/probes necessary to amplify
    * Usually kept at a lower concentration

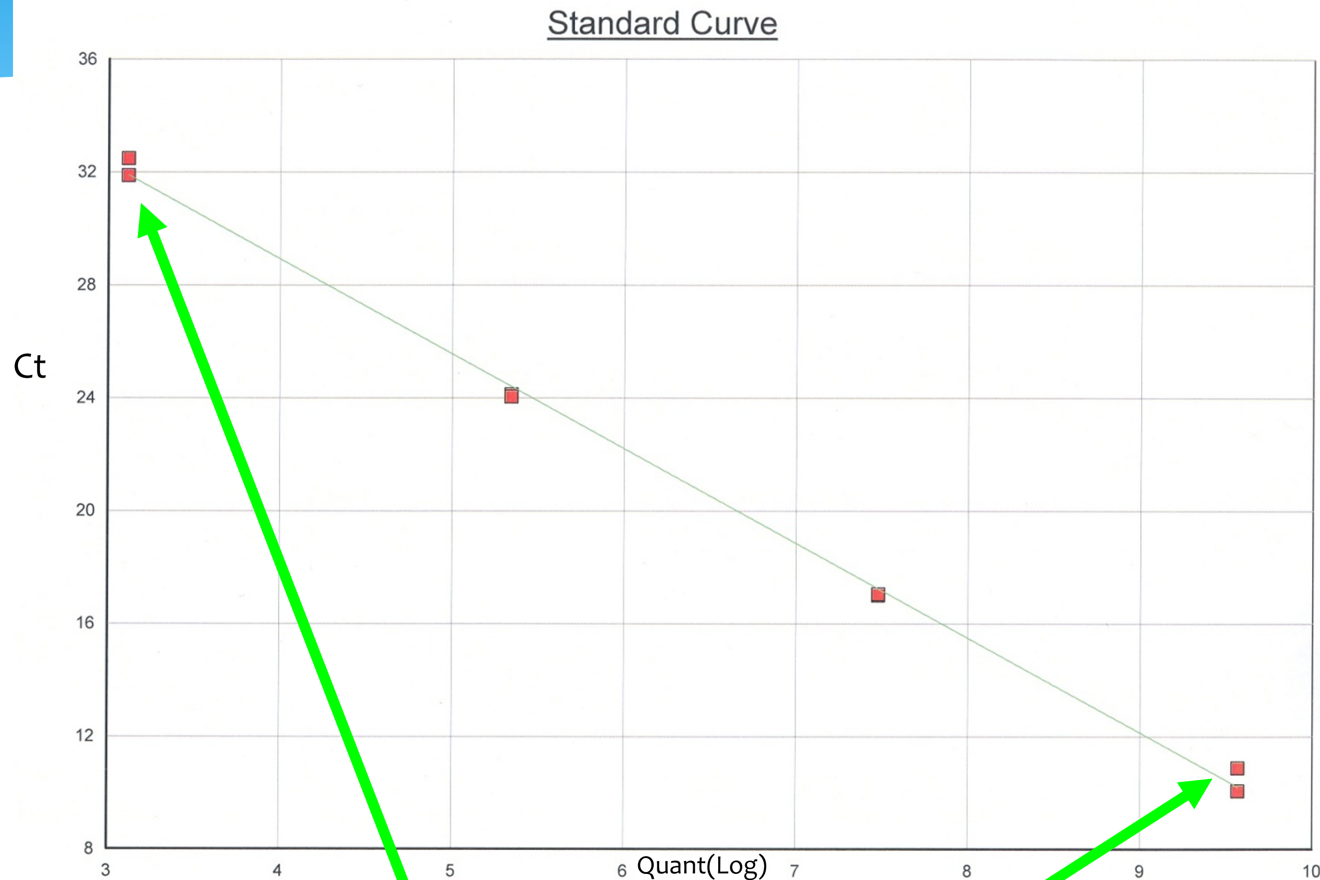


Assay Linearity

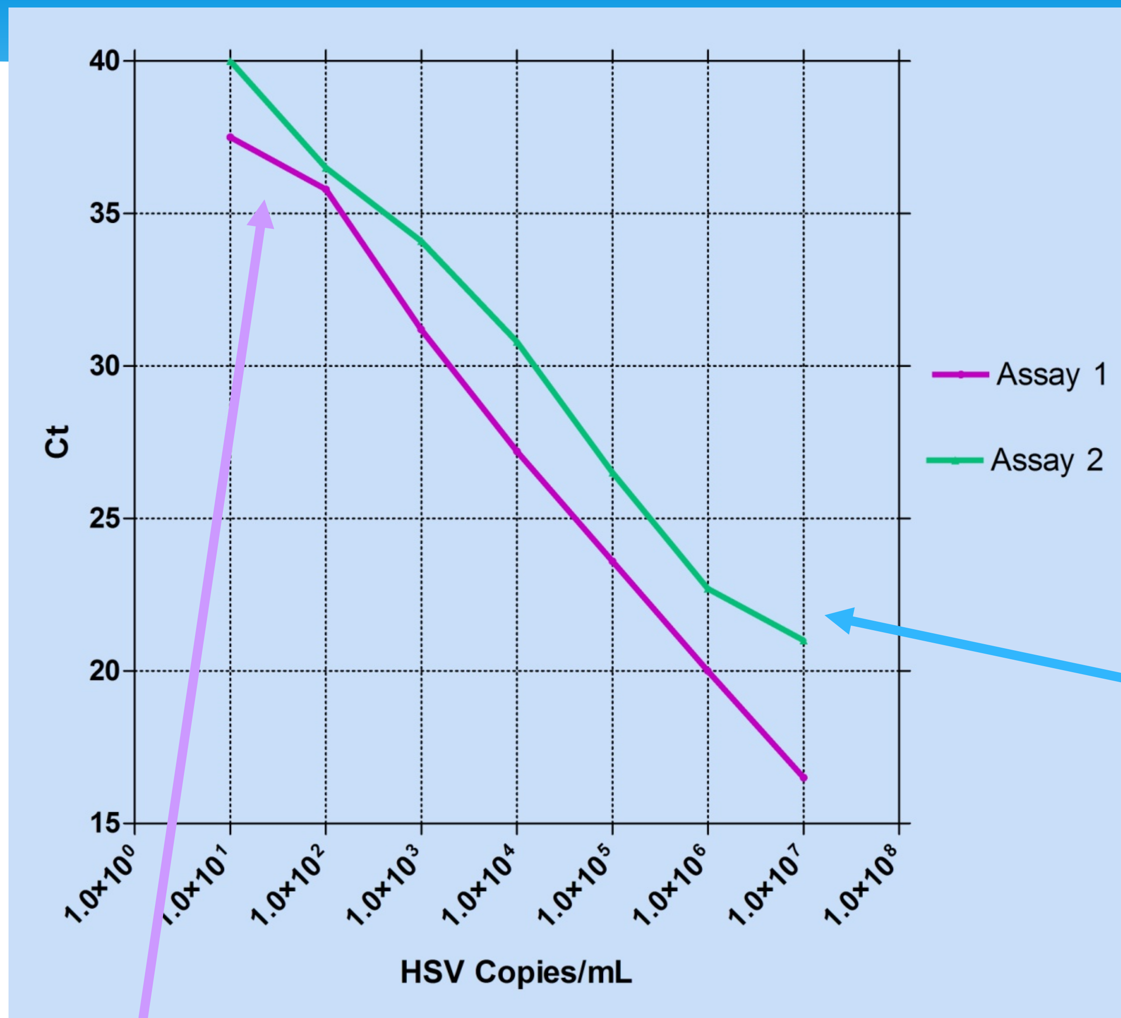
Extraction

PCR Assay

Linearity of the Assay



At High & Low Viral Quants –
Enough primer, probe ?
Extraction Efficiency



Non-linear at the top

Non-linear
at the bottom

Usual to have 6-7 logs measuring range

Assay Sensitivity

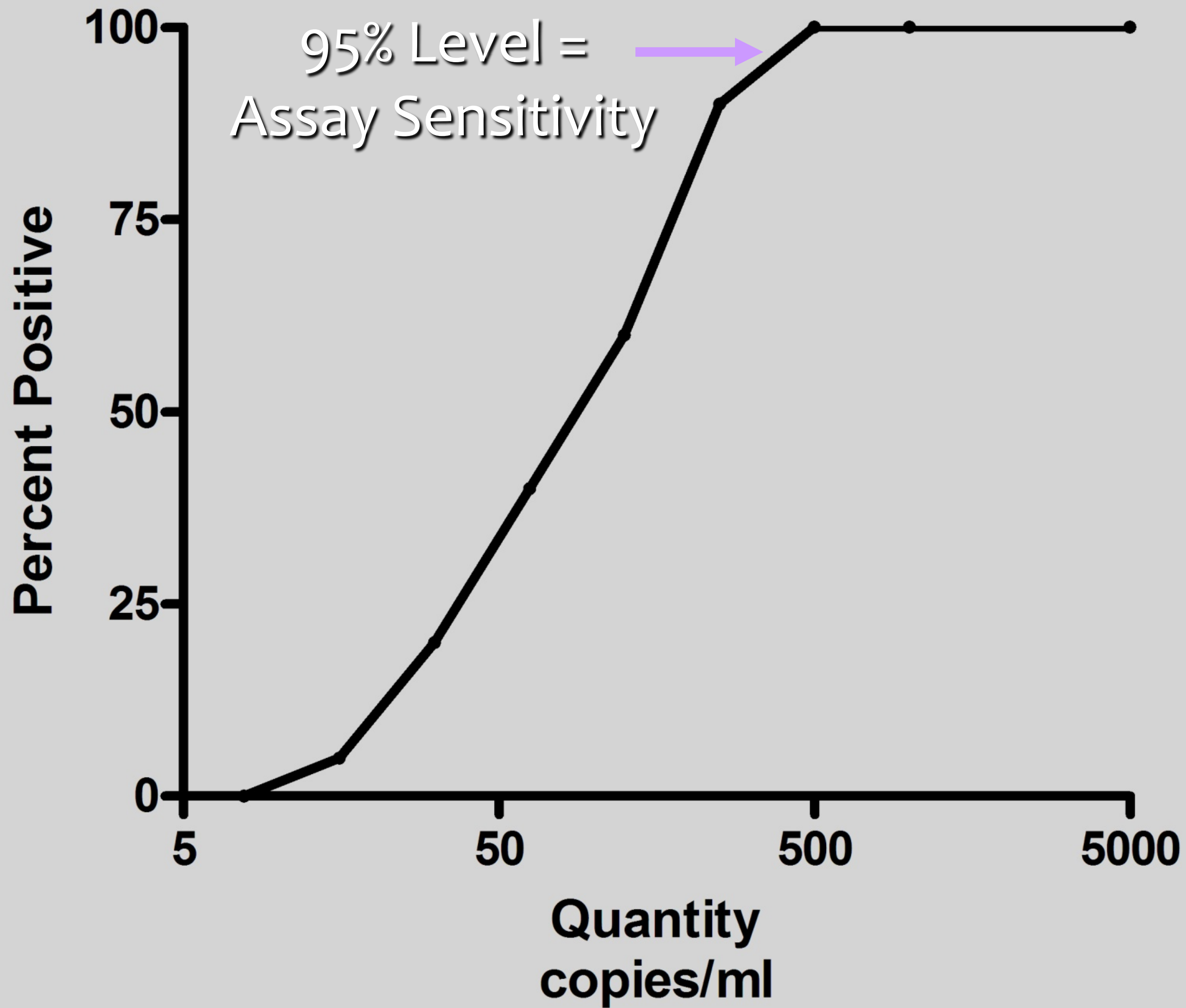
Assay Sensitivity – Extraction + PCR

* Linearity + Probability
  * May detect 1 virus in well
  * Can you get the virus into the well?

  * Example –
    * Start with 200 uL serum
    * Elute purified DNA into 100 uL buffer
      * [2 fold concentration – if assume 100% yield]
    * Use 10 uL in the PCR assay

Start with 25,000 c/ml virus
- **Or 5,000 copies (start)**
- **After elution have 5,000 c/100 ul**
- **500 copies into the PCR assay**

Start with 250 c/ml virus
- **Or 50 copies (start)**
- **After elution have 50 c/100 ul**
- **5 copies into the PCR well**



Serial two-fold dilutions – at least 6 replicates

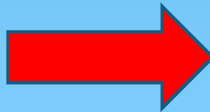
Example – Assay Sensitivity Determined Utilizing a Probit Statistical Analysis

Dilutions	# Run	Quant c/mL	Pt 1 # Pos	Quant c/mL	Pt 2 # Pos
Neat	6	1,799	6	1,011	6
1:2	6	961	6	437	6
1:4	6	727	6	197	6
1:8	6	309	6	148	6
1:16	6	120	6	72	4
1:32	6	109	6	60	4
1:64	6	32	5	46	4

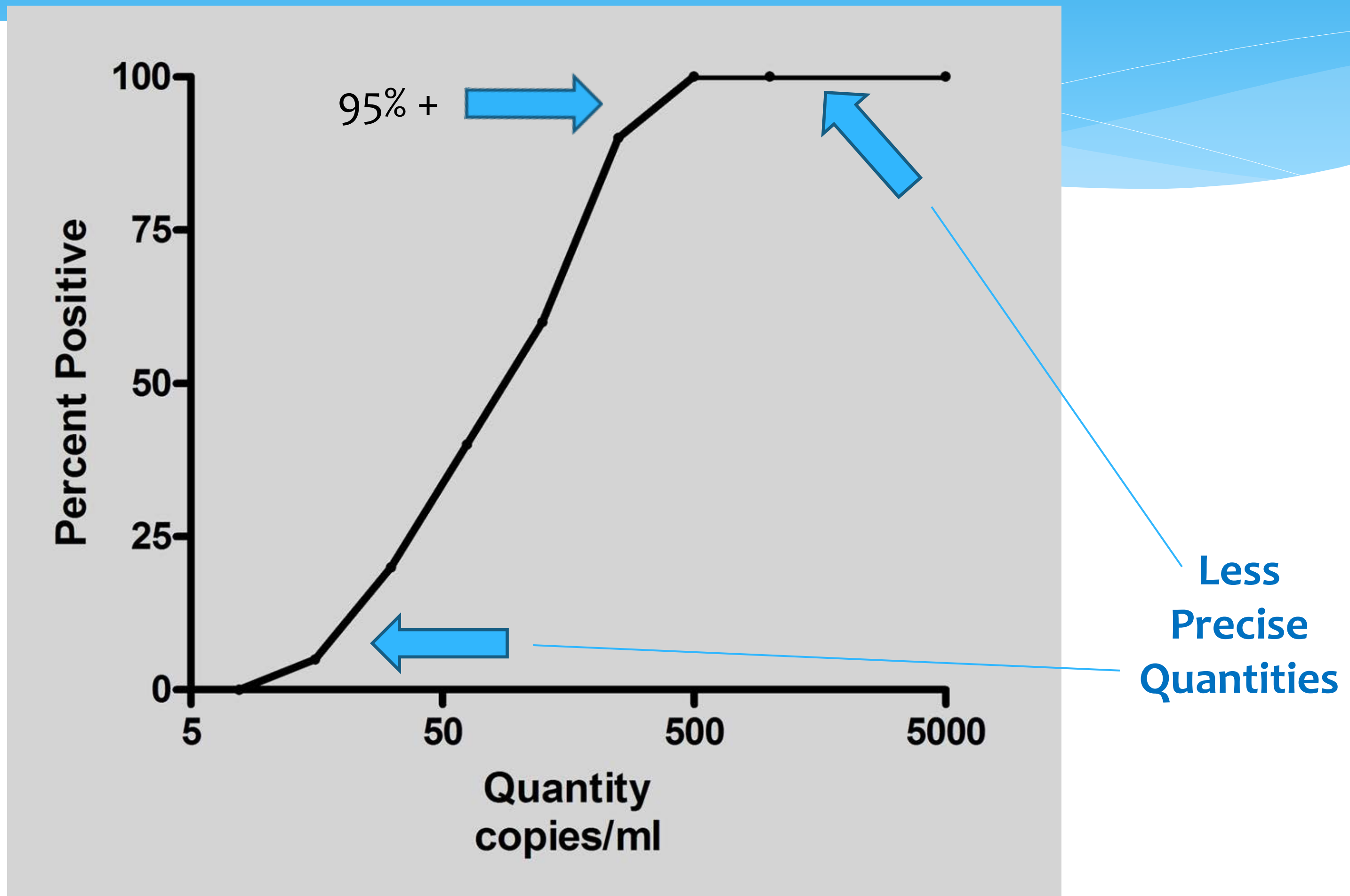
95% Level about 100 and 140 in these 2 samples

Precision and Accuracy

Sensitivity of the Assay
Serial Measurements



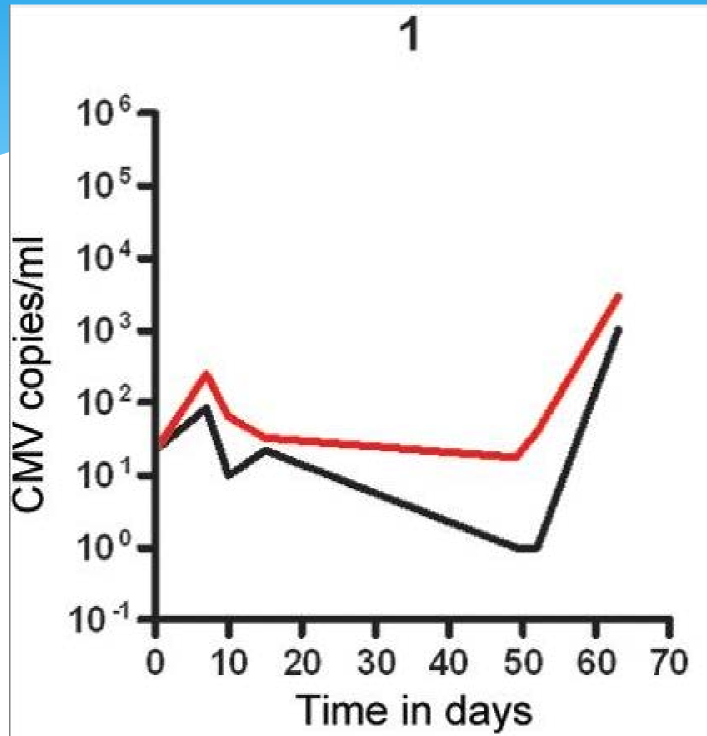
Assay Imprecision



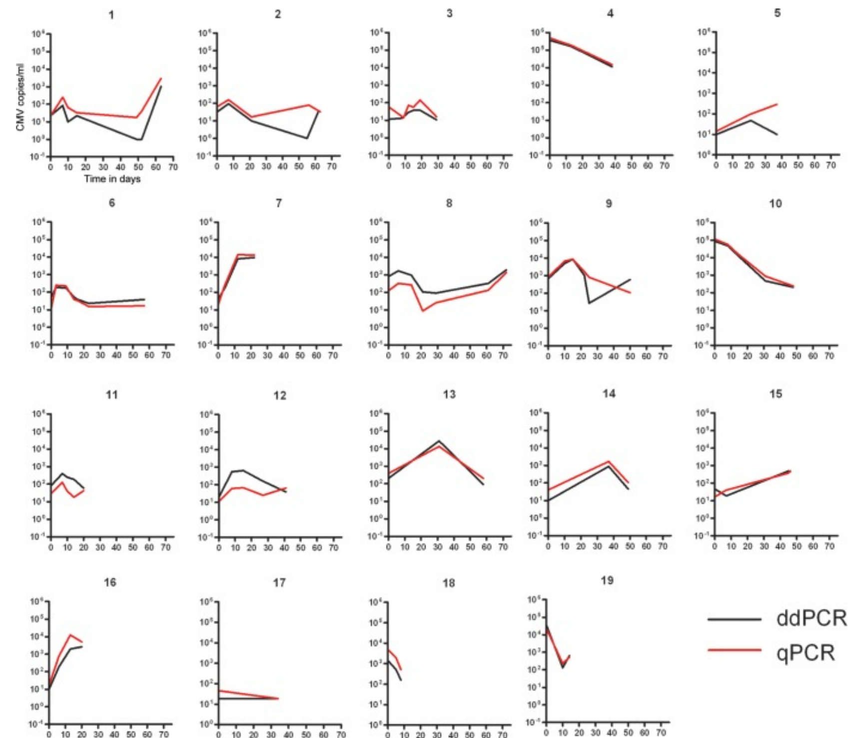
Less
Precise
Quantities

Limit of Detection vs Limit of Quantification

Reproducibility – 19 Patients
CMV qPCR vs ddPCR



No difference between Methods and Clinical Response Time (flair detection no earlier)



Sedlak, et al J. Clin Microbiol 2014:52:2844

CMV Specific Information

Standards & Commutability

Primers &Probes

Available CMV Standardization Materials

* 1st WHO International Standard for Human CMV
  * NIBSC 09/162
  * 5.0 x 10e6 IU/mL
  * Needs Extraction
* NIST Standard – SRM 2366a CMV DNA (TowneΔ147 BAC)
  * 1.8 x 10e6 Copies/mL
  * Purified DNA material (PCR control only)
* Secondary Reference Materials
  * Quantitative Panels – 3 commercial sources
    * Needs Extraction
  * Purified, Quantified Virus
    * DNA material (PCR control only)
* **Limited quantity so can't be used for routine testing**

Routine Use of Standard Curves

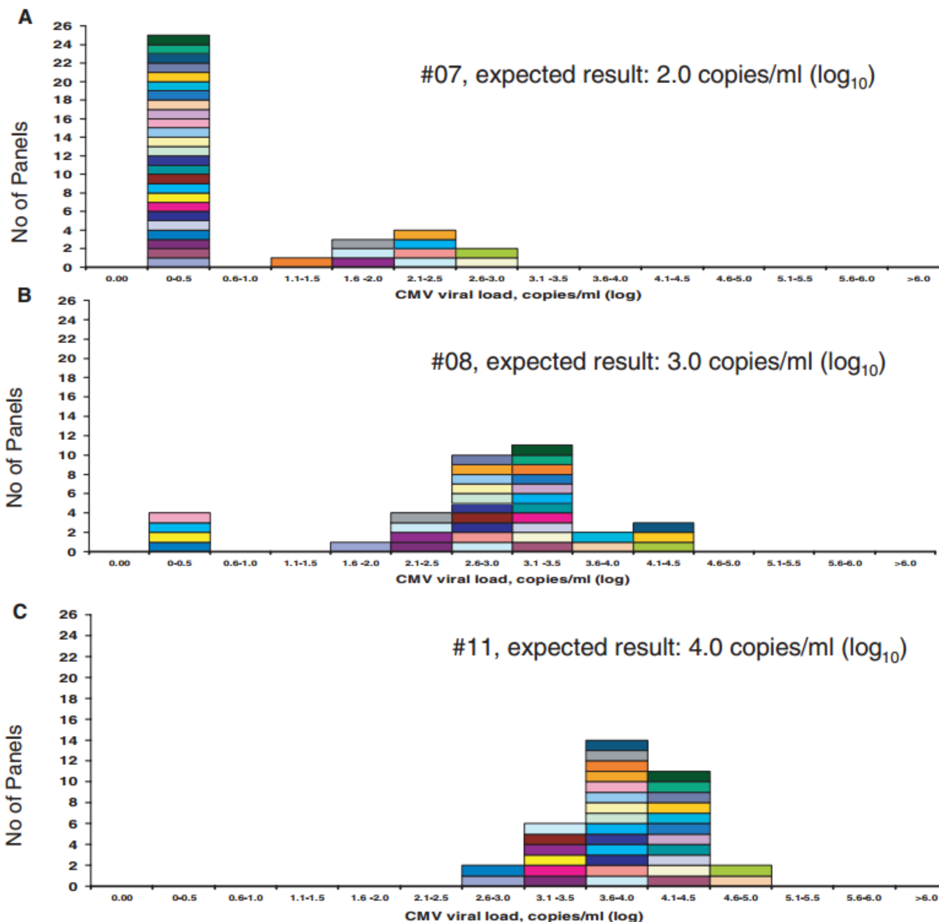
* Frequency
  1. Instrument Software – Stored Standard Curve
     * Calibrate 1 time, then use for the duration of that lot of mix
     * Current CLIA/CAP guidelines require "Calibration Verification" checks at least every 6 months
  2. If not, then run a standard curve with every run
     * Plasmid Clone Preparations
       * Either amplicon or larger area of sequence

Standards Purpose

1. Assign the “correct value” to your standard materials
2. Assure that the value doesn’t change over time within a single lab
3. Assure that the same values are obtained across methods and across multiple labs

Pre-WHO Standard Between-Lab Comparison -33 labs

Pang, et al 2009

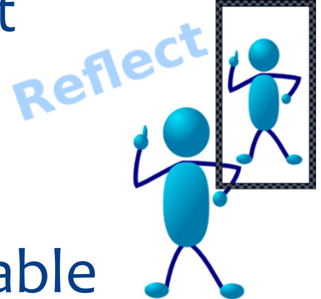


Wide variation of results, both above and below a commonly used Clinical Cutoff of 1,000 Copies/mL

Post WHO Release ??

“Commutability”

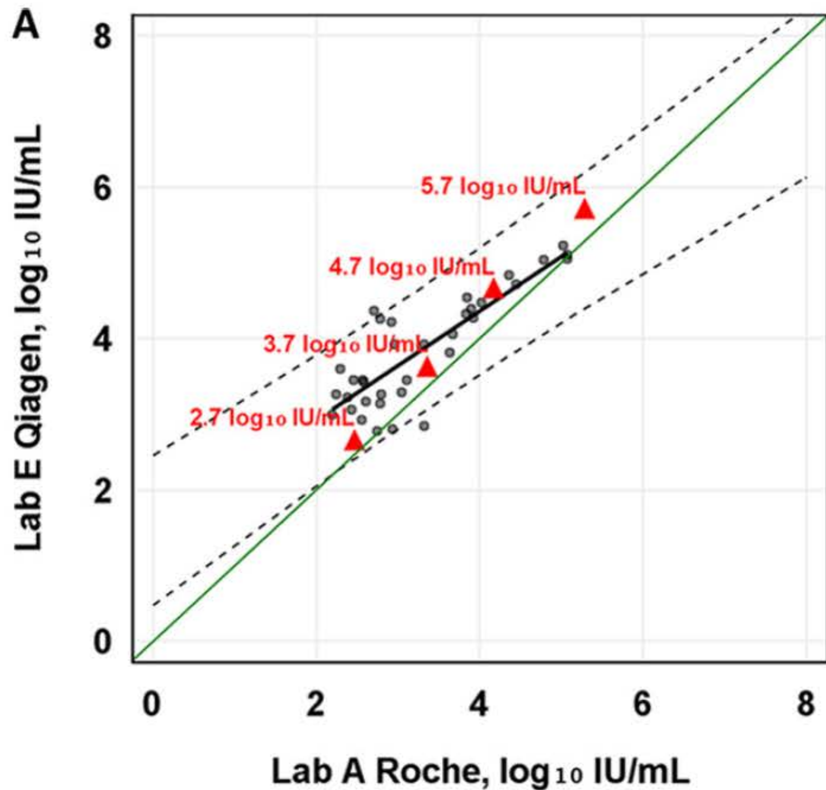
1. A RM would be considered commutable when a measurement procedure produces the same result for a RM as it does for an authentic patient sample that contained the same analyte concentration.

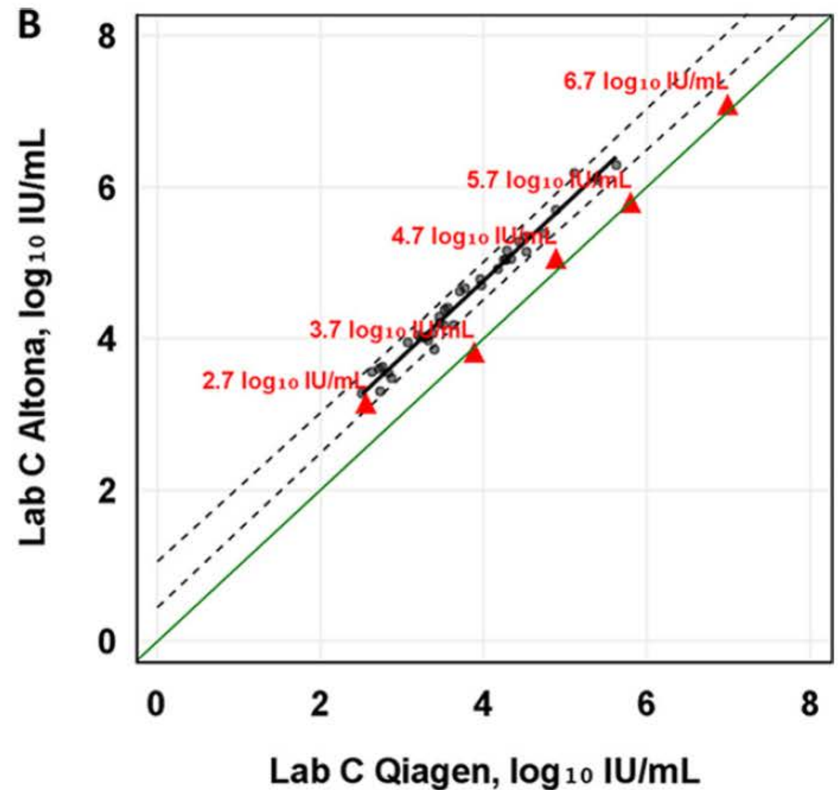


2. Measurement procedures calibrated with commutable RMs will produce results for clinical samples that are equivalent among all procedures, i.e. the results are traceable to the reference system and there is no calibration bias among the measurement procedures.

Commutability Evaluation Data

Hayden, 2015 (40 positive samples, 6 labs/methods)





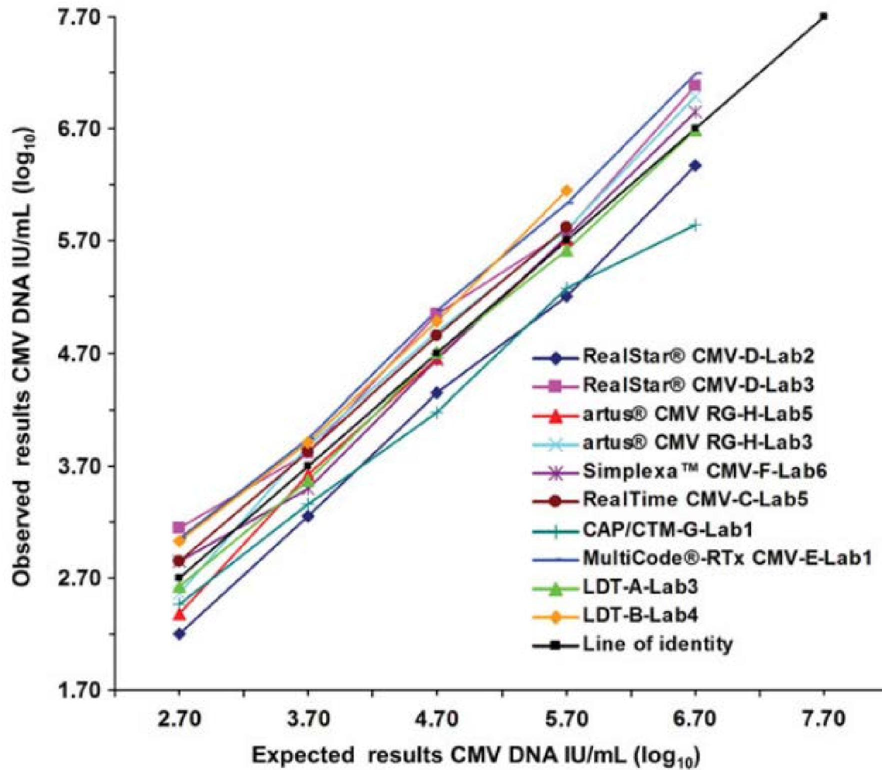
Cross Lab & Method Commutability

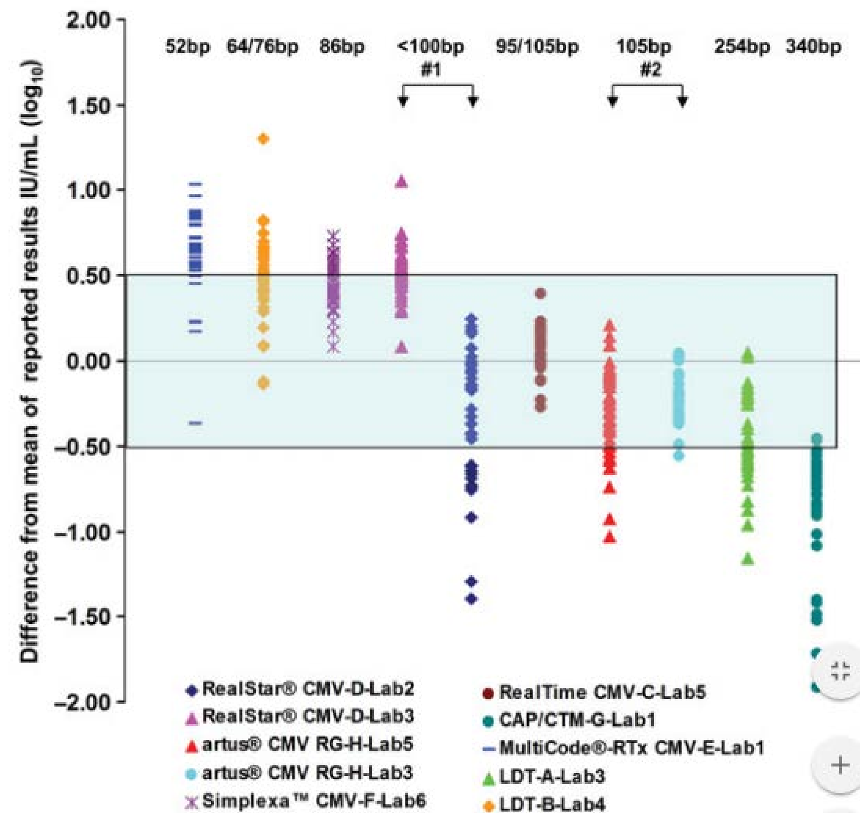
Hayden, RT et al JCM 2013

	Lab A Luminex	Lab A Roche	Lab B Altona	Lab C Altona	Lab C LDT	Lab C Qiagen	Lab D LDT	Lab E Abbott	Lab E Qiagen
Lab A Roche	3/4								
Lab B Altona	4/4	4/4							
Lab C Altona	4/4	3/5	4/4						
Lab C LDT	2/4	5/5	4/4	3/5					
Lab C Qiagen	0/4	4/5	4/4	1/5	4/5				
Lab D LDT	3/3	4/4	3/3	4/4	4/4	2/4			
Lab E Abbott	0/3	4/4	3/3	2/4	3/4	3/4	4/4		
Lab E Qiagen	2/3	4/4	3/3	3/4	4/4	4/4	4/4	4/4	
Lab F Focus	4/4	3/4	4/4	4/4	2/4	0/4	3/3	0/3	0/3

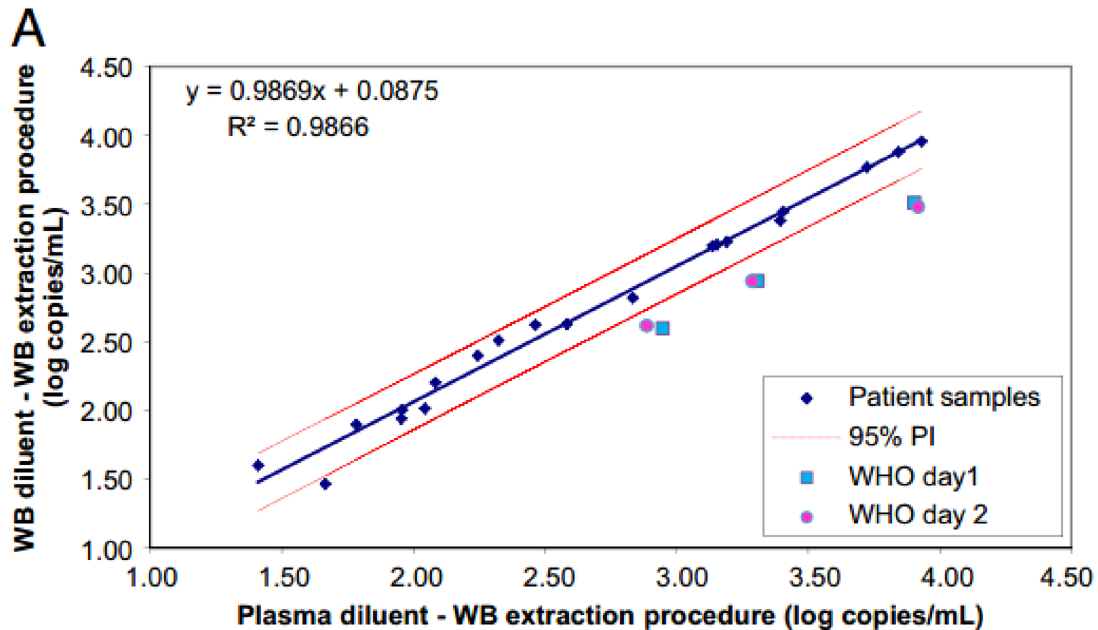
Commutable | Marginally commutable | Not commutable

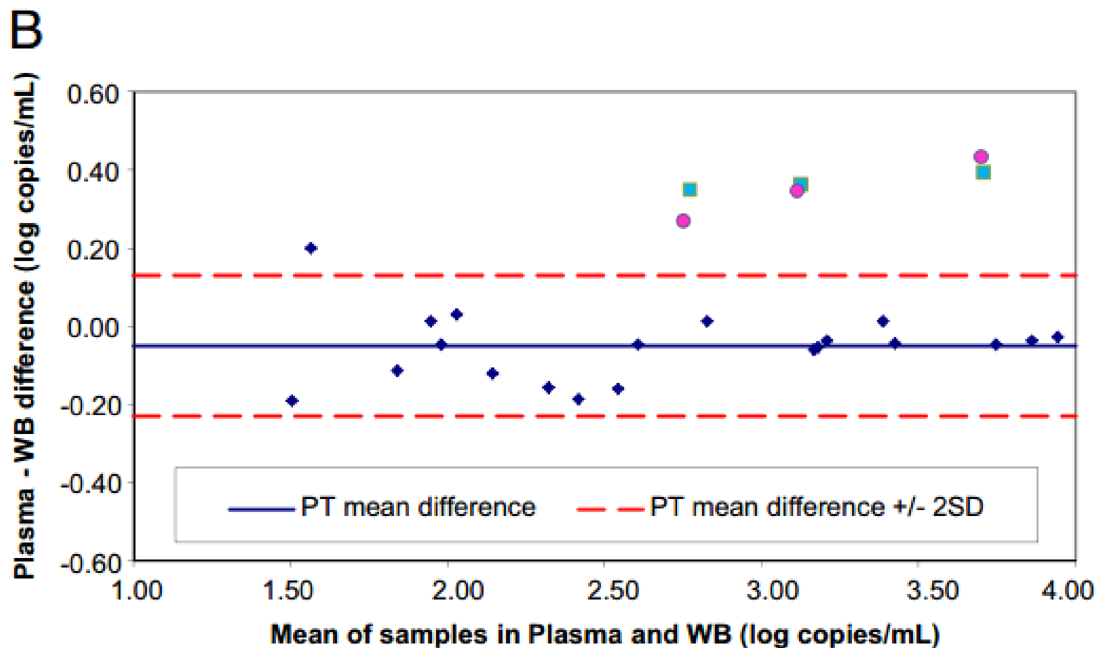
Preiksaitis, et al 2016





Jones, et al 2016





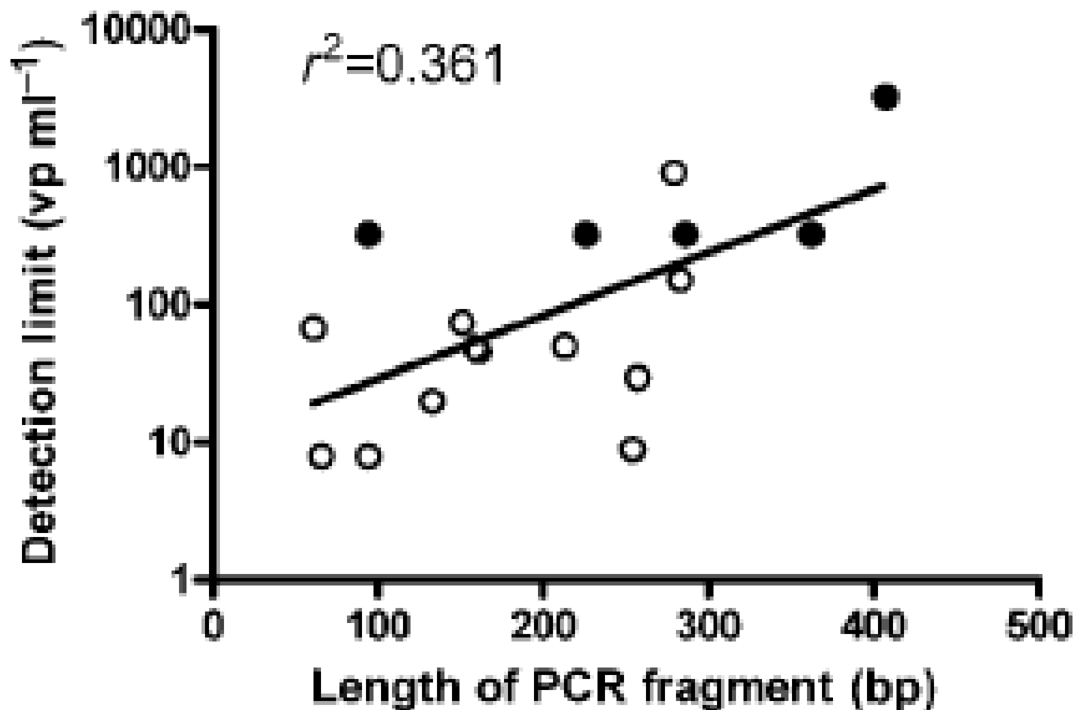
CMV WHO Standard lack of commutability in Whole Blood

Primer and Probe Design

CMV Primer and Probe Design

* Habbal, W, Monem, F, and Gartner, BC. – 2009
* Identified 57 papers describing 82 primer pairs
* 17 Selected for further evaluation based on absence of mismatching with CMV sequences in GenBank.
* Evaluated all for their assay sensitivity
  * 5 immediately eliminated due to lack of sensitivity
  * Best sensitivity was seen with 3 primer sets in gB region
  * Also found… ..

Primer/Probe Design



Detection levels highest for the smallest primer sets

Primer Selection and Standardization

Mannonen 2014

Comparison of Two CMV-PCR Tests

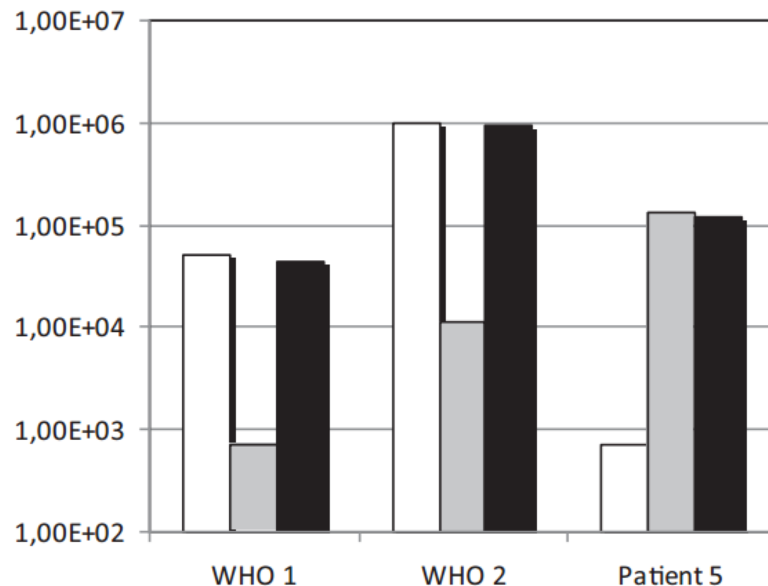


Fig. 4. Impact of the G to T mutation in the binding region of forward primer. PCR mixes containing 900 mM of the normal forward primer (white bars), the G to T modified forward primer (gray bars) and a mix of both (450 mM each; black bars) were used to amplify the patient sample (patient 5). Two dilutions of the CMV WHO standards amplified with the normal forward primers were used as quantification standards (WHO 1: 1×10^6 IU/ml, WHO 2: 5×10^4 IU/ml). The impact of the mutation on the quantification of the DNA extract from the patients and of the WHO standard is approximately 2-log.

Forward Primer Mismatch resulted in a significant decrease in Amplification for the patient sample

Current FDA Approved CMV Quant Tests

* Roche Cobas Ampliprep/Cobas Taqman Assay
  * Solid Organ transplant monitoring – July 2012
  * Stem Cell transplant monitoring – May 2016
* Qiagen CMV RGQ MDX Kit (Artus)
  * Qiagen EZ1 Extraction Instrument
  * Roto-Geen Q MDX Instrument
  * Solid Organ transplant monitoring – June 2014
* Nothing currently available for other clinical CMV infections {Diagnosis??}

Questions?