*Vira*Quant™: Development and evaluation of a quantitative multiplexed viral load assay for CMV, EBV, HHV6, HHV7, and BKV

Garcia EP[1], Ford D[1], Donahue J[1], Wells P[1], Diankov V[1], Yen-Lieberman B[2] and Slepnev V[1]. [1]Primera Biosystems, Mansfield, MA and [2]The Cleveland Clinic, Cleveland, OH

ABSTRACT

Background: *Vira*Quant™ is a new multiplexed quantitative PCR assay for CMV, EBV, HHV6, HHV7 and BKV. *Vira*Quant™ is based on STAR technology (*Scalable Target Analysis Routine*) a novel approach that integrates qPCR and capillary electrophoresis (CE) allowing for the quantitative measurement of multiple targets in a single sample with high sensitivity (Garcia et al, J Mol Diagn 7:444, 2005). Multiplexing offers simultaneous analysis of quantitative standards for each virus and a DNA extraction control with each sample to enhance analytical performance and clinical confidence.

Materials and Methods: Specific DNA primers are designed for each viral genome in the *Vira*Quant™ assay so that fragments amplified from each target are unique in size. Standards and extraction controls are similarly structured with distinct lengths. Aliquots of the reaction are removed after successive PCR cycles and separated by CE. As in conventional qPCR, targets are quantified based on reconstructed amplification curves. We developed the *Vira*Quant™ assay on the ABI-3730 DNA Analyzer and examined its' analytical and clinical performance.

Specificity was studied by the analysis of DNA from a wide range of potential interfering targets. We established the dynamic range for each virus by analysis of up to 3,000,000 copies per reaction. The precision of the *Vira*Quant™ system was assessed in a multi-parameter experiment at 4 levels of each target on 4 instruments with 4 operators over 2 months (n=28).

A small patient cohort (n=19), on whom molecular CMV analysis had been ordered, was analyzed for each viral target. Sensitivity and specificity for CMV was calculated relative to the hybrid capture reference method. The distribution of viral load other than from CMV in these subjects was also noted.

Results: The linear dynamic range for each viral target was 500 – 3,000,000 copies/mL plasma. There was no detected reaction from other herpes and polyoma viruses. The median total precision (%CV) of the measured copy number was 19.2% and ranged from 8.2 to 24.2%.

Clinical sensitivity for CMV was 85% and specificity was 100% against the results of the hybrid capture method. Viral DNA from at least one of the other pathogens was found in 75% (6/8) of the patients who were negative for CMV by *Vira*Quant™.

Conclusion: The *Vira*Quant™ assay system employs STAR technology to enable simultaneous PCR measurement of the viral load of CMV, EBV, HHV6, HHV7 and BK virus. The assay shows excellent analytical performance. A study on a small cohort of patients shows good concordance with a reference CMV method and highlights the potential importance of viruses other than CMV and prospects for co-infection. The *Vira*Quant™ assay allows for the consolidation of the viral load tests for these five key pathogens into a simplified, cost-effective method.

INTRODUCTION

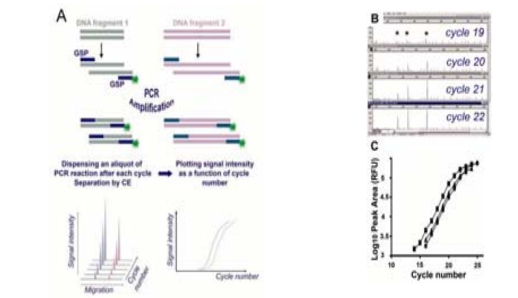
Introduction of effective immunosuppressive drugs in the 1980's has had a profound impact on the success of organ transplants. Post-transplant immunosuppressive therapy is complex and usually includes a combination of drugs and approaches based on a patient's individual situation and the organ transplanted. Side effects from drug-induced immunosuppression are common, including infectious disease complications. Nearly half of new transplant patients are diagnosed with infections, typically viral, in the first 3 to 6 months. These viral infections can pose grave consequence for transplant patients including allograft rejection. Viral infections can result from transmission from the donor tissue, exposure to the environment or reactivation of the patient's own latent viruses. The viruses of most concern to transplant physicians vary with the organ transplanted, although certain viruses such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are of universal concern. Whereas the polyoma viruses such as BKV, (HHV) 6 and 8 can be problematic for kidney transplant patients.

We have developed *Vira*Quant™, a STAR technology based nucleic acid diagnostic that simultaneously detects and quantifies the levels of five viral targets critical to the transplant patient. STAR combines the desirable traits of both real-time PCR (precision, sensitivity and quantification) and DNA microarray (multiplexing) into a single system. STAR represents an innovative integration of real-time multiplex PCR and capillary electrophoresis (CE), allowing the simultaneous quantitative measurement of multiple targets in a single sample with high sensitivity. Because CE allows accurate size determination of fluorescently labeled nucleic acids from 50 to 1000 bases with the single base resolution, assays can be developed for dozens of targets whose identities are defined by the specific size of its corresponding PCR product, while maintaining quantification capabilities equal to or better than those observed with established real-time PCR methods. There are certain practical considerations such as primer design that will contribute to the upper limits of multiplexing for the STAR technology. STAR is fast, cost-effective, and has a large dynamic range.

The *Vira*Quant™ assay has been designed to quantify viral load of CMV, EBV, BKV, HHV6 and HHV7 from either plasma or whole blood samples. We have determine that the Limit of Detection is ~20 copies/reaction. The lower and upper Limits of Quantification range from 60 copies/reaction at the low end to > 3,000,000 copies/reaction, respectively. We also present data that demonstrates that several serum and blood components and drugs commonly used in immunocompromised individuals do not significantly affect the assay. Further studies demonstrate that the presence of DNA isolated from related viruses or a cohort of nosocomial microorganisms do not interfere *Vira*Quant**™**. Analysis of serially diluted samples harboring all five viral targets demonstrate excellent linearity from 500 to 3,000,000 copies/mL plasma. Precision studies demonstrate >30% CVs from 250 to 500,000 copies/reaction. Finally, a small study that compares determination of CVM viral load as determined by Hybrid Capture or *Vira*Quant**™**, demonstrates 91% clinical sensitivity and 100% specificity. Finally, 83% of samples that were negative for CMV as determined by hybrid capture were determined to be positive for at least one additional viral target as determined by *Vira*Quant**™** demonstrating utility.

Description of STAR Technology

Figure 1. Diagrammatic Representation of STAR Technology


A
DNA fragment 1
DNA fragment 2
GSP
PCR Amplification
Dispensing an aliquot of PCR reaction after each cycle Separation by CE
Plotting signal intensity as a function of cycle number
Signal intensity
Migration
Cycle number
B
cycle 19
cycle 20
cycle 21
cycle 22
C
Log10 Peak Area (RFU)
Cycle number

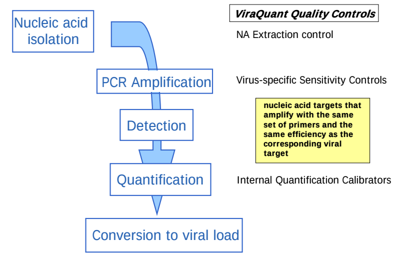
(A) Diagrammatic representation of the STAR process. (B) Electropherograms derived from sequential multiplex PCR cycles. Peaks representing arc, homer1a, and zif268 are marked with an asterisk. Small repeating peaks represent DNA molecular size markers. (C) Amplification curves for arc (closed triangles), homer1a (open circles) and zif268 (closed squares) were reconstructed by plotting the area under each peak against cycle number.

Description of *Vira*Quant™

Multiplex measure of DNA load of five viral targets in a single reaction CMV, EBV, HHV6, HHV7 and BKV

- **Highly sensitive and specific for each viral target**
- **Consolidates testing into a single platform**
- **Enables a rapid turnaround time**

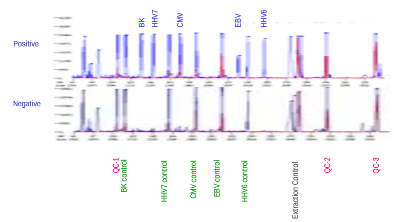
Figure 2. Overview of ViraQuant process


Nucleic acid isolation
PCR Amplification
Detection
Quantification
Conversion to viral load
ViraQuant Quality Controls
NA Extraction control
Virus-specific Sensitivity Controls
nucleic acid targets that amplify with the same set of primers and the same efficiency as the corresponding viral target
Internal Quantification Calibrators

Quality control features built into *Vira*Quant™

DNA extraction control
Internal quantification calibrators (QC-1,-2,-3)
Virus-specific sensitivity controls

Figure 3. Typical Data generated by ViraQuant



*Vira*QuantTM: Analytical Validation

Figure 4. ViraQuant™ Precision ***ViraQuant™ shows excellent precision demonstrating %CVs between 8 and 24% between copy numbers of 250 and 500,000 per reaction.*** Samples were prepared that were spiked with all five viral targets at varying levels (A). All levels were represented in triplicate and all viral targets were seeded at the highest and lowest target level. Samples were processed in *Vira*Quant™ on 5 consecutive days to examine precision. Coefficients of variation were calculated based on input copy number. %CV plotted again copy number is shown (B).

A Target Input Levels per reaction

RXN Well	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
BK	0	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	31,250	31,250	31,250	977	977	977	61	61	61
V7	0	1,000,000	1,000,000	1,000,000	500,000	500,000	500,000	15,625	15,625	15,625	488	488	488	61	61	61
CMV	0	1,000,000	1,000,000	1,000,000	250,000	250,000	250,000	7,813	7,813	7,813	244	244	244	61	61	61
EBV	0	1,000,000	1,000,000	1,000,000	125,000	125,000	125,000	3,906	3,906	3,906	122	122	122	61	61	61
HHV6B	0	1,000,000	1,000,000	1,000,000	62,500	62,500	62,500	1,953	1,953	1,953	61	61	61	61	61	61

B

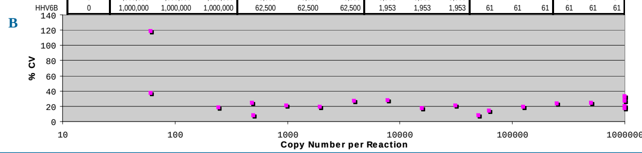


Figure 5. Interfering Substances and Specificity ***(A) ViraQuant™ is not inhibited by compounds commonly found in plasma samples.*** A list of potentially interfering substances was developed based on common medications taken by kidney transplant patients, endogenous substances that may be elevated in any individual, and anticoagulants or preservatives that may be encountered in excess due to a short draw or the like. All twenty substances were dissolved in DI water, ethanol, or DMSO depending on their solubility. Compounds were added to a plasma sample (pool of 80 different donors) that was spiked with viral targets. CMV, EBV and HHV6 viral particles were added at 25,000 or 2,500 copies/mL while HHV7 and BK was added as genomic DNA at 25,000 or 2,500 copies/mL. Individual controls were produced containing the same amount of each of these solvents. N=12. Each substance's response was compared to the response of the control sample diluted with the same solvent and the difference between responses was calculated.

(B) ViraQuant™ does not cross-react with either related organisms or common microorganisms present in immunocompromised individuals. In a similar manner, plasma samples were spiked with a mixture of genomic DNA equivalent to 1 x 10^8 genome equivalents derived from the microorganisms listed in the table in a background of 25,000 copies/mL of CMV, EBV, HHV6, BK and HHV7 in (A).

A Interfering Substances

Compound	Diluent	Test Concentration (mg/L)	Delta CT (with and without compound addition)				
acetylsalicylic acid	water	600	BK	HHV7	CMV	EBV	HHV6
captopril	water	5		2.5 x 10^4 copies/mL			
metoprolol	water	5	-0.31	-0.22	-0.16	-0.35	-0.28
sodium azide	water	0.50%		2.5 x 10^3 copies/mL			
gancyclovir	water	10	-0.34	-0.05	-0.12	0.38	0.35
amoxicillin	DMSO	75					
bilirubin, conjugated (mixed)	DMSO	50		2.5 x 10^4 copies/mL			
indomethacin	DMSO	36	-0.02	-0.05	0.10	-0.23	-0.06
methotrexate	DMSO	160		2.5 x 10^3 copies/mL			
nifedipine	DMSO	0.4	0.08	-0.15	-0.36	-0.37	-0.33
FK-506	DMSO	0.5					
cyclosporinA	DMSO	14					
fenofibrate	Ethanol	45					
hydrochlorothiazide	Ethanol	6					
hydrocortisone	Ethanol	0.69		2.5 x 10^4 copies/mL			
ibuprofen	Ethanol	500	-0.12	-0.25	-0.26	-0.34	-0.44
lovastatin	Ethanol	53		2.5 x 10^3 copies/mL			
naproxen	Ethanol	500	-0.04	-0.10	0.17	-0.24	0.45
salicylic acid	Ethanol	200					
prednisone	Ethanol	3					

B Specificity

Organism	Delta Ct (with and without organisms) BK	CMV	EBV	HHV6B	HHV7
Steptococcus pneumoniae	-0.54	-0.77	-0.93	-0.75	-0.59
Neisseria meningitidis					
Streptococcus pyogenes					
Borrelia burgdorferi					
Campylobacter jejuni					
Staphylococcus aureus					
Candida albicans					
Listeria Monocytogenes					
Varicella Zoster Virus (HHV3)					
HSV-1					
HSV-2					
HHV-8					
SV-40					
JCV					

Figure 6. Limit of Blank ***ViraQuant™ does not detect the presence of CMV, EBV, BKV, HHV6 or HHV7 viral DNAs in serum samples derived from apparently normal healthy donors.*** Fifty serum samples from an apparently healthy donor population were evaluated with the *Vira*Quant™ assay. Samples were chosen that had been pre-screened for antibody titers to CMV and EBV so that an adequate population of donors that may harbor virus was screened. Four hundred microliters of each sample was processed for genomic DNA and screened.

Immune Status	No. of serum samples	*Vira*Quant™ Results CMV	EBV	BK	HHV6	HHV7
CMV IgG+	25	ND	ND	ND	ND	ND
CMV IgG-	25	ND	ND	ND	ND	ND
EBV IgG+	25	ND	ND	ND	ND	ND
EBV IgG-	2	ND	ND	ND	ND	ND

Figure 7. Limit of Detection/ Limit of Quantification

Twenty replicates of plasma matrix containing 435, 250, 100, 75, 50, 25, and 12.5 copies/reaction of CMV, EBV, HHV6, HHV7 and BK were assessed in the *Vira*Quant™ assay. Copy number and %CV will be calculated for each data set. The concentration at which targets are detected in 75% of samples is defined as the LOD. The concentration at which the %CV is > 40% is the Lower LOQ, *N=20*

Target	copies/reaction LOD	Lower LOQ
CMV	20 copies	65 copies
EBV	25 copies	75 copies
BK	20 copies	60 copies
HHV6	50 copies	90 copies
HHV7	20 copies	75 copies

Figure 8. Linearity ***ViraQuant™ shows good linearity between 500 and 3,000,000 copies/mL plasma for CMV, EBV, BKV, HHV6 and HHV7*** "High" viral target samples were created by spiking plasma matrix pools to yield a result 20 to 30% beyond the expected linear range of the assay (1,000,000 viral copies/mL). The "high" viral target samples were prepared by spiking plasma matrix with either purified viral particles (CMV, EBV, HHV6) or purified genomic DNA (BK, HV7). Beginning at 3,000,000 copies/mL, the high viral target sample were serially diluted in half-log steps to a final concentration of 500 copies/mL.. The relative concentration (X – level number) versus the mean measured concentration (Y) is plotted for each sample. The data was fit to a polynomial regression and the number of levels (corresponding to the concentration range) included in the regression was decreased systematically until the non-linear coefficients in both a second and third order polynomial are not significant within a 95% confidence interval (p ≥ 0.05). The polynomial regressions was completed using Analyze-It software. The high and low levels/concentrations at which the non-linear coefficient is found not to be significant is considered the linear range according to that particular sample.

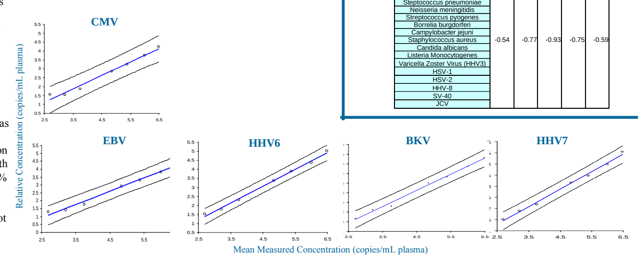


Figure 9. ViraQuant™: Clinical Proof of Concept **Clinical samples obtained from The Cleveland Clinic Foundation were screened in *ViraQaunt™*.**

	Hyb Capture	ViraQuant				
Tube no.	CMV	CMV	BK	EBV	HHV6B	HHV7
1	1896	1600				
2	3252	4400				
3	neg	neg			3200000	
4	neg	neg				
5	806	3700				
6	1787	940				
7	neg	neg		7300		860
8	453	580		3000		detected*
10	3300	detected*				
11	2768	5300				
12	2700	2300				
13	neg	neg		54000		
14	1357	neg		detected*		
15	neg	neg			10000	10000
16	19000	6700				
20	28000	16000	25000			
21	2500	neg				
22	1030	6900				
23	neg	neg			42000000	

*Vira*Quant™ data compared to CMV Hybrid Capture
- 85% Sensitivity
- 100% Specificity
- Samples that were negative for CMV in both assays were positive for another virus in the *Vira*Quant™ panel.

83% of samples that were negative for CMV in both assays were positive for another virus in the *Vira*Quant™ panel. *Vira*Quant™ ~ 25% of CMV positive samples were coinfected with at least one other virus in the *Vira*Quant™ panel.

Conclusions

- *Vira*Quant™ is highly sensitive and specific for detection and quantification of five viral targets: CMV, EBV, BK, HHV6 and HHV7
- Precision studies demonstrate better than 30% CVs over a wide dynamic range: 250 and 500,000 copies/reaction
- *Vira*Quant™ shows excellent linearity from 500 to 3,000,000 copies/mL plasma
- Not affected by compounds commonly found in patient blood samples
- No cross reactivity to common human pathogens or other closely related viruses
- *Vira*Quant™ shows excellent clinical sensitivity (86%) and specificity (91%) compared to CMV Hybrid Capture assay