ViraQuant: Development and evaluation of a quantitative multiplexed viral load assay for CMV, EBV, HHV6, HHV7, and BKV

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ABSTRACT

Background: A new assay, ViraQuant, has been developed that simultaneously quantifies the five viruses of primary interest in transplant patients. The assay gains its advantages from the Scalable Target Amplification Routine (STAR) platform, which allows for the quantitative measurement of multiple targets in a single sample with high sensitivity, specificity and precision. The purpose of this study was to demonstrate the performance characteristics of this assay.

Methods: Analytical performance (specificity, precision, dynamic range and limits of detection and quantification) was examined according to the Clinical and Laboratory Standard Institute guidelines (CLSI). A small patient cohort (n=35), tested for CMV infection was assessed using the ViraQuant assay. Sensitivity and specificity for CMV was calculated relative to the hybrid capture reference method..

Results: The precision (%CV) of the measured copy number ranged from 10 to 35% on copy number with a linear dynamic range of 500 to 1,000,000 copies/mL. As few as 20 copies/reaction were detectable with a lower quantification limit as low as 60 copies/reaction. No significant interference was detected with a host of related microorganisms or the presence of common substances that may be found in blood products. A number of cases of co-infection with multiple viruses were detected even in small patient group.

Significance: The ability to consolidate testing for these pathogens into a simplified, cost-effective method with excellent analytical and clinical performance can expedite assessment of the risks of viral disease, identify the presence of unsuspected co-infections helping to improve patient outcomes.

ViraQuant: The next generation multiplex for clinical diagnostics

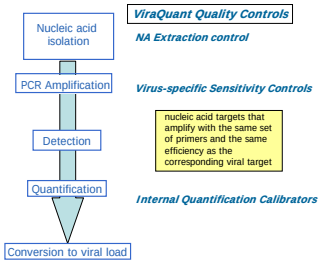
Introduction of effective immunosuppressive drugs in the 1980's has had a profound impact on the success of organ transplants. Post-transplant immunosuppressive therapy is complex and usually includes a combination of drugs and approaches based on a patient's individual situation and the organ transplanted. 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 results 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. HHV6 and HHV7 can cause viral disease in transplant recipients or complicate the course of CMV and EBV diseases. BK has been implicated in PVAN resulting in loss of the graft.

The ViraQuant assay has been designed to quantify viral load of CMV, EBV, BKV, HHV6 and HHV7 from either plasma or whole blood samples. The results of analytical verification of ViraQuant detection of each of the viral targets is presented. Figure 1 describes the method and technology.

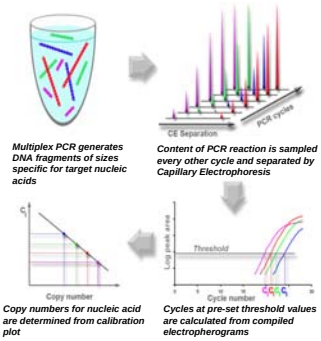
Figure 1. The ViraQuant assay

We have developed ViraQuant, 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.

A. Overview of ViraQuant process



B. Overview of STAR technology



Multiplex PCR generates DNA fragments of sizes specific for target nucleic acids

Content of PCR reaction is sampled every other cycle and separated by Capillary Electrophoresis

Copy numbers for nucleic acid are determined from calibration plot

Cycles at pre-set threshold values are calculated from compiled electropherograms

C. Representative electropherograms

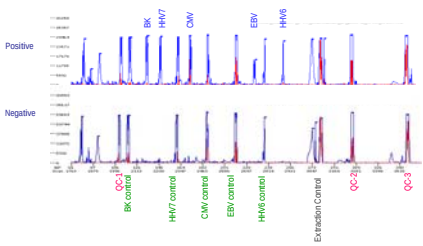
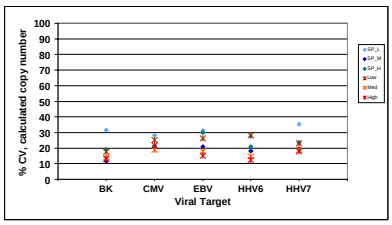


Figure 1. ViraQuant Description. (A) Flow of sample processing through ViraQuant to viral load determination. Note that a number of controls are included in the assay to monitor nucleic acid extraction, PCR amplification and quantification *for each individual sample*. (B) Overview of STAR technology. (**C**) Representative electropherogram depicted the amplicons detected from a mock clinical sample that is positive for all 5 viral targets (upper panel) and a mock clinical sample that is negative for all 5 viral targets. For each of the viral targets, a sensitivity control is present at ~25 copies per reaction. Presence of sensitivity controls indicate successful amplification with each of the primer pairs. Quantification controls (QC-1, QC-2, QC-3) are seeded at 250, 2,500 and 25,000 copies per reaction. Amplification from the Extraction control provides confidence of adequate nucleic acid extraction during processing of clinical samples. Sizes of amplicons range from 112 to 350 bases.

Figure 2. ViraQuant demonstrates excellent precision for all viral targets

A



ViraQuant shows excellent precision demonstrating %CVs ON COPY NUMBER between 10 and 35%. Samples were prepared that were spiked with all five viral targets at 2000, 48,000 or 100,000 copies/mL plasma, processed on the EasyMag (Biomerieux) and analyzed in ViraQuant. A duplicate set of samples that did not undergo extraction was also included to monitor reproducibility in the absence of sample extraction. Reproducibility was monitored in duplicate, twice a day over 20 days as described by CSLI 5A. (A) Graphical depiction of total variability for each of the viral targets. (B) Total and within run %CV for each viral target based on detected **COPY NUMBER** is shown.

B

	BK		*CMV*		*EBV*		*HHV6*		*HHV7*	
	Total	Within Run	Total	Within Run	Total	Within Run	Total	Within Run	Total	Within Run
SP_LOW	31.6	28.9	28.1	20.7	31.2	22	27.5	18.5	35.2	27.3
SP_MED	11.6	7.7	20.9	7.1	20.8	6.8	18.2	8.7	19.6	8.7
SP_HIGH	19.1	6.5	21.4	6.5	30.1	7.4	20.8	6.1	23.5	7.4
LOW	17.6	15.9	25.2	19.1	26.2	22.6	28.3	22	23.2	17.7
MED	14.1	8.2	19	9	17.8	9.5	14.5	10	19	9.8
HIGH	13.1	7.5	21.7	13.1	15.2	10.6	12.3	6.4	17.9	9

Figure 3. Specificity and Absence of Interference

The ability of ViraQuant to detect and quantify each of the 5 viral targets in the presence of competing DNAs or substances commonly found in blood samples of transplant patients was assessed. (A) For specificity, 1e8 genome equivalents/mL plasma of a collection of related viruses (herpesviruses and polyomaviruses), nosocomial microorganisms and other pathogens common to transplant patients were spiked into samples containing 2,000, 48,000 or 100,000 copies/mL of all 5 ViraQuant viral targets. Control samples spiked with only ViraQuant viral targets were also prepared. Twelve replicates of each condition was tested. Samples were processed on the EasyMag and analyzed by ViraQuant. Analysis showed that there was no inhibition of detection of the ViraQuant viral targets as assessed by CT with a shift of ≤ 0.5. **(B)** Similarly, spiking of plasma samples containing 2,000, 48,000 or 100,000 copies/mL of all 5 ViraQuant viral targets with potentially inhibitory substances also did not inhibit detection of the viral targets. Concentrations were taken from CSLI 7A. For substances that were not specified by the guidelines, 10X the Cmax value was added.

A. Specificity

Group1	Group2
Steptococcus pneumoniae	HSV1
Neisseria meningitidis	HSV2
Streptococcus pyogenes	HSV3 (VZV)
Clostridium perfringens	HHV8
Borrelia burgdorferi	JC (polyomavirus)
Clostridium Difficile	SV40 (Simian vacuolating virus 40)
Campylobacter jejuni	Human T-lymphotropic virus (type II)
Staphylococcus aureus	HBV (Hepatitis B)
Enterococcus faecalis	Papillomavirus_HHPV_11
Candida albicans	Papillomavirus_HHPV_16
Pseudomonas aeruginos	Papillomavirus_HHPV_6B
Listeria Monocytogenes	Papillomavirus_HHPV_18
Mycoplasma	

B. Potentially interfering substances tested

Substance	Conc (mg/L)	Substance	Conc (mg/L)
acetylsalicylic acid	600	naproxen	500
Captopril	5	lovestatin	53
metoprolol	5	salicylic acid	600
sodium azide	0.50%	prednisone	3
gancyclovir	10	triglycerides	5000
amoxicillin	75	cholesterol	1000
bilirubin, conjugated (mixed)	50	albumin, human	13,240
indomethacin	36	ceruloplasmin	600
methotrexate	160	genomic DNA	4ug
nifedipine	0.4	hemoglobin	5000
FK-506	0.5	heparin, lithium	30000U/L
cyclosporin A	14	niacin	0.5
fenofibrate	45	IgG, human	15,000
hydrochlorothiazide	6	IgM, human	15,000
hydrocortisone	0.69	Anti-DNA antibodies	4
ibuprofen	500		

Absence of multiplex cross-talk among the 5 viral targets.

Viral targets were assessed in ViraQuant in pairs where one was seeded at 500,000 copies and a second at 500 copies per reaction. Each viral target was screened against all viruses targeted in ViraQuant. Detection and quantification of the virus seeded at 500 copies per reaction was not effected.

Figure 4. Limits of Detection and Quantification

Limit of detection (LOD) was determined for each viral target assessed by ViraQuant according to CSLI EP17A. Sixty replicates of plasma samples spiked with varying levels of all 5 viral targets were sample processed and assessed in ViraQuant. The LOD was determined to be the concentration demonstrating 95% detection. Values are shown in the recovered copy number per reaction. The limit of quantification (LOQ) shown for each target was determined from the same data set based on %CV of $\leq 35\%$.

	copies/reaction	
Target	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 5. Linearity

ViraQuant shows linearity from 500 to 1,000,000 copies viral target/mL of plasma. Plasma samples Were spiked with all 5 viral targets ranging from 100 to 3,000,000 per mL And extracted using the EasyMag. Samples were then assessed for viral load with ViraQuant. Data analysis was performed as directed in CSLI EP6-A. For each of the viral targets the spiked concentration (X) versus the mean measured concentration (Y) is plotted.

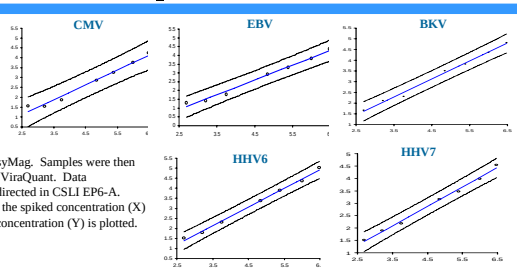


Figure 6. ViraQuant enables rapid detection of multiple infections simultaneously

The ViraQuant assay has been designed to quantify viral load of CMV, EBV, BKV, HHV6 and HHV7 from either plasma or whole blood samples. The results of analytical verification of ViraQuant detection of each of the viral targets is presented. A small study that compares determination of CVM viral load as determined by Hybrid Capture or ViraQuant, demonstrates 86% clinical sensitivity and 95% specificity. Of samples that were negative for CMV, 66% were determined to be positive for at least one additional viral target demonstrating the importance of multiplex detection.

Sample Name	CCF_DiGene_CMV	CMV	BK	HHV7	EBV	HHV6
1_CCF_Sample1	1210	899				
1_CCF_Blood_sample2	474	Neg		291	520	
5_CCF_Blood_sample3	Neg	Neg				
2_CCF_Blood_sample4	Neg	Neg	145	178		
2_CCF_sample5	700	387		168		
3_CCF_sample6	9406	1154		105		
3_CCF_Blood_sample7	972	1124		143	2207	
4_CCF_Blood_sample8	Neg	Neg				
5_CCF_Blood_sample9	Neg	Neg		456		
6_CCF_Blood_sample10	Neg	Neg			3798	
4_CCF_sample11	522	134				
5_CCF_sample12	3648	478			332	102
7_CCF_Blood_sample13	3648	243			708	
6_CCF_Blood_sample14	Neg	Neg				
7_CCF_Blood_sample15	Neg	Neg				
6_CCF_sample16	272600	79275				
7_CCF_sample17	Neg	Neg				
8_CCF_Blood_sample18	Neg	Neg		226		
8_CCF_sample20	748	702		119		
2_CCF_sample22	665	Neg		152		
4_CCF_sample25	905	510	1998	870	387	289
3_CCF_Blood_sample26	Neg	606	131		404	180
5_CCF_sample27	1969	1042				
4_CCF_Blood_sample28	Neg	Neg		173		
6_CCF_sample30	2635	1927	138			
7_CCF_sample31	Neg	Neg				
6_CCF_Blood_sample32	Neg	Neg		565		
7_CCF_Blood_sample33	Neg	Neg				
8_CCF_Blood_sample34	Neg	Neg		179		
8_CCF_sample35	Neg	Neg			268	
1_CCF_Blood_sample36	Neg	Neg		1106		
2_CCF_Blood_sample37	Neg	Neg		116		
3_CCF_Blood_sample38	Neg	Neg	29016		34003	
4_CCF_Blood_sample39	Neg	Neg				
1_CCF_sample40	Neg	Neg		130		

SUMMARY of ViraQuant

- Quantitative measurement of CMV, BKV, EBV, HHV-6, HHV-7
- Sensitive detection
- Large linear dynamic range
- Excellent precision
- Built-in controls

ViraQuant enables multiplexed viral monitoring helping to improve patient management