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Multicenter Comparison of Laboratory Performance in Cytomegalovirus and Epstein-Barr Virus Viral Load Testing using International Standards

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Abstract

Background—Infections with cytomegalovirus (CMV) and Epstein Barr Virus (EBV) remain important in solid organ transplantation. Quantitative viral nucleic acid testing is a major advance to patient management. These assays are limited by a lack of standardization, resulting in viral load measurements that differ among clinical laboratories. The variability in viral load measurements makes interpretation of multicenter clinical trials data difficult. This study compares the current practices in CMV and EBV viral load testing at four large transplant centers participating in multicenter Clinical Trials in Organ Transplantation (CTOT/CTOTC).

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Disclosures

JAF of MGH and GS of Washington University were members of the Scientific Advisory Board of PrimeraDx, Inc. Assay systems developed by that company were not used at either site as part of this study. GS is a member of the Roche Diagnostics Advisory Board. All other authors report no potential conflict of interest.

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Methods—Viral load testing was performed on well-defined viral preparations according to standard operating procedures at each site.

Results—Among centers, CMV viral load testing was accurate compared to WHO International Standards and within acceptable variation for this testing method. EBV viral load data were more variable and less accurate despite the use of international standards.

Conclusions—These data suggest that comparison of CMV, but not EBV, viral load measurements at these sites is possible using current assays and control standards. Standardization of these assays is facilitated by using the WHO International Standards and will allow comparison of viral load results among transplant centers. Assay standardization must be performed prior to initiation of multicenter trials.

Keywords

Cytomegalovirus; Epstein-Barr virus; viral load; quantitative nucleic acid testing; WHO standards; clinical trial

Introduction

In transplant recipients, viral load testing has become the primary modality for diagnosing active disease due to cytomegalovirus (CMV) and Epstein Barr Virus (EBV) infections and monitoring responses to therapy (1–7). Using whole blood or plasma, viral nucleic acid is extracted and subjected to amplification using quantitative, real-time polymerase chain reaction (qPCR) based assays. The number of viral copies present in the initial sample is determined by comparison to a set of standards with known copy number. Until recently, there were no standardized materials for preparing these sets of standards. Thus, laboratories developed and validated testing protocols using calibrators that may or may not have been equivalent. Further, although the methodologies used for viral load testing may be similar from one laboratory to another, reagents, extraction methods, primers, and amplification platforms can vary from one clinical laboratory to another (8–14). This further contributes to assay variability making it problematic to compare results between laboratories (10,11).

Quantitative viral load testing for CMV and EBV provides a method to assess the intensity of immunosuppression and protocol safety in the setting of clinical trials, notably for studies of new immunosuppressive regimens or antiviral therapies. Given the lack of assay standardization, the interpretation of study data in terms of development of viral load cutoffs for the clinical diagnosis and management of CMV or EBV infection or EBV-associated post-transplant lymphoproliferative disorders (PTLD) has been challenging. The World Health Organization (WHO) has developed assay standards for both CMV and EBV (15,16). These viral preparations are intended for use by laboratories and manufacturers to calibrate secondary reference materials, so that the concentration of virus in a sample can be expressed and compared in international units. The Clinical Trials in Organ Transplantation (CTOT) and the Clinical Trials in Organ Transplantation in Children (CTOT-C) are research consortia sponsored by the National Institute of Allergy and Infectious Diseases (NIAID) that conduct clinical trials and associated mechanistic studies to improve outcomes in adult and pediatric organ transplantation. Given that viral monitoring is a routine component in

the care of transplant recipients, this study was designed to compare the accuracy and variability of CMV and EBV viral load testing using the WHO viral standards in comparison to commonly used commercially available viral panels at four of the CTOT transplantation centers.

Materials and Methods

Study Design

CMV and EBV viral load testing was performed at four independent clinical laboratories from academic medical centers that are members of the Clinical Trials in Organ Transplant (CTOT) Mechanistic Studies Working Group (Cleveland Clinic, Emory Transplant Center, Massachusetts General Hospital, and Washington University School of Medicine). Each laboratory performed the assays according to center-specific standard operating procedures. All samples were tested blindly. Results were reported in copies per milliliter (ml). The procedural characteristics of the assay(s) used at each site are shown in Table 1 and described below. One site used two separate protocols for testing. All studies were approved by institutional review panels for human studies at each participating clinical center.

Quantitation Panels

For each virus and study site, one commercial panel and one panel created from the WHO International Standards were tested. These panels were chosen to represent the control materials that were already in use for verifying CMV and EBV test systems. They also allow the entire process from nucleic acid extraction to quantitation to be assessed since they consist of viral particles suspended in a plasma matrix. The CMV commercial panel consisted of a single replicate of five members from the OptiQuant CMV_{tc} Panel from Acrometrix (Life Technologies, Grand Island, NY) and a negative control consisting of dialyzed, defibrinated human plasma (BaseMatrix; SeraCare, Milford, MA). The OptiQuant CMV_{tc} panel consists of human plasma containing CMV strain AD169. The manufacturer specified that the concentration of CMV DNA in each of the panel members was 500, 5000, 50,000 and 500,000 copies/ml. The CMV International Standard panel consisted of triplicate tenfold serial dilutions of the 1st WHO International Standard for Human Cytomegalovirus obtained from NIBSC (code 09/162; Hertfordshire, England). This preparation consists of lyophilized CMV Merlin strain and was assigned a potency of 5×10^6 IU/ml based on a worldwide collaborative evaluation in which the consensus value was 5×10^6 copies/ml (15). The lyophilized standard was reconstituted in 1 ml of nuclease free water and dilutions were prepared in dialyzed, defibrinated human plasma (BaseMatrix; SeraCare, Milford, MA) to achieve panel members spanning 50 to 500,000 copies/ml. A negative control consisting of BaseMatrix alone was included.

The EBV commercial panel consisted of a single replicate of all six members of the OptiQuant EBV Plasma Panel obtained from Acrometrix (Life Technologies, Grand Island, NY). This panel includes human plasma containing EBV ranging in concentration from 1000 to 10,000,000 copies/ml and a negative control. The EBV International Standard panel consisted of triplicate tenfold serial dilutions of the 1st WHO International Standard for EBV obtained from NIBSC (code 09/260; Hertfordshire, England). This preparation contains

lyophilized EBV strain B95-8 and was assigned a potency of 5×10^6 IU/ml based on a worldwide collaborative evaluation in which the consensus value was 5×10^6 copies/ml (16). This standard was prepared in the same manner as the CMV International Standard and included panel members spanning 10 to 1,000,000 copies/ml and a negative control.

For all four panels, each panel component was assigned a code and relabeled to anonymize the source and remove any indication of the expected copy number. The quantitation panels were simultaneously frozen at -80 °C and shipped overnight on dry ice to each study site.

Viral Load Assays

Each laboratory performed CMV and EBV viral load testing on each of the panels as per institutional standard protocols for plasma as indicated in Table 1. One site performed two separate assays for both CMV and EBV testing including one standard and one multiplex assay system.

All of the extraction protocols involve sample lysis under denaturing conditions in the presence of protease. For protocols 2 and 4 the initial sample volume was 200ul. For protocol 1 initial sample volume was 263ul. For protocol 3, the initial sample volume was 500ul for CMV and 1000ul for EBV. For protocol 5, the initial sample volume was 200ul. In protocols 1, 2, and 4, nucleic acids are adsorbed onto a silica membrane, washed, and eluted into buffer. In protocols 3 and 5, magnetic beads rather than a silica membrane are used. The exact makeup of the reagent buffers is proprietary, but differs among the protocols. The elution volumes for protocols 3 and 4 were 60ul. For protocol 1, 2, and 5 the elution volume was 83ul, 50ul, and 100ul respectively.

The amplification and detection protocols are all based on real-time quantitative detection using fluorescent dyes linked to oligonucleotide probes. Protocols 1, 2, and 3 used the same amplification and detection kit, although on different thermocycling instruments (Table 1). Protocol 4 used the IcePlex system, which is a multiplex real-time PCR and capillary electrophoresis instrument. Protocol 5 used a lab-developed protocol and reagents on an ABI 7500 Real Time System. The volume of nucleic acid used in protocols 1, 2, and 3 was 20ul. Protocol 4 and 5 used an input volume of 10ul and 5ul for amplification.

Statistical Methods

Each viral load result was \log_{10} transformed before analysis. Negative results and any result reported as positive but below the reportable range were not included in the calculation of mean and range.

Results

Testing Protocols

For CMV and EBV viral load testing, two quantitation panels for each virus were sent to each of the four transplantation sites for testing. All five protocols used automated nucleic acid extraction systems with either silica-membrane or magnetic bead based isolation of the viral DNA. However, the sample volume, elution volume, reagents, and extraction platforms differed for each protocol (Table 1). For CMV, three of the protocols targeted the Major

Intermediate Early (IE) gene using the Qiagen artus CMV reagent kit, and amplification and detection was performed on three separate thermocycler platforms. The other two protocols (Primera Dx ViraQuant and a laboratory developed assay protocol) utilized primers targeting the US28 and UL54 genes. The reportable range for these protocols varied considerably among sites. For one assay, the lower limit for quantitation was 50 copies/ml while another assay had a lower limit of 2000 copies/ml. Similarly for EBV, three protocols used the same Qiagen artus™ EBV reagent kit with primers that target the EBNA1 protein. Protocol 5 also targeted the EBNA1 gene, while Protocol 4 targeted EBNA-LP. The reportable ranges for these assays also varied with a limit of detection for one assay of 25 copies/ml while another produced quantitative data above 4,000 copies/ml. This information shows the absence of standardization among these sites in terms of the protocols, reagents, and extraction, and amplification systems being used.

CMV

The qualitative and quantitative performance of CMV viral load testing was compared using two different sample panels covering the typical range of concentrations assessed in clinical laboratories using human plasma containing known amounts of CMV or serial dilutions of the WHO International CMV Standard. There were no false positive results reported for the negative control from either panel (Table 2). The results from the commercial panel included a single result using Protocol 5 (see Table 1), in which no viral DNA was detected in the specimen expected to contain $2.7 \log_{10}$ copies/ml (500 copies/ml) which is below the expected lower limit for Protocol 5. CMV was detected using all five protocols in samples expected to contain greater than $3.7 \log_{10}$ copies/ml (5000 copies/ml), although one result was not quantifiable. All five protocols provided quantitative results for the commercial panel samples expected to contain 4.7 and $5.7 \log_{10}$ copies/ml (50,000 and 500,000 copies/ml). The mean viral load measured at each of these concentrations was lower than the expected value. The difference between the mean reported value and the expected value at each concentration varied from 0.44 to $0.54 \log_{10}$ copies/ml. As shown in Figure 1, individual results for the commercial panel were all below the expected value. Eight of the 20 samples (40%) fell within $\pm 0.5 \log_{10}$ copies/ml of the expected value, which is considered the acceptable degree of variation for quantitative nucleic acid tests (10). Only two of the five assays gave results within $0.5 \log_{10}$ of the expected value at every concentration tested. At those concentrations where quantitative results were reported, the difference between the highest viral load result and the lowest viral load result was within or close to $0.5 \log_{10}$ copies/ml (Table 2).

For the CMV WHO International Standard panel, virus was not detected in the majority of samples expected to contain 0.7 or $1.7 \log_{10}$ copies/ml (5 or 50 copies/ml). CMV was detected in all of the samples expected to contain $2.7 \log_{10}$ copies/ml (500 copies/ml). Quantitative results were obtained on all but one sample expected to contain $3.7 \log_{10}$ copies/ml (5000 copies/ml). For those replicates with quantitative data, the mean viral load was lower than the expected value at all concentrations and varied from 0.09 to $0.4 \log_{10}$ copies/ml. As shown in Figure 1, the majority (39 of 60, 65%) of the individual results were below the expected value and all fell within $\pm 0.5 \log_{10}$ copies/ml. When data were compared among protocols, the difference between the highest quantitative result and the lowest

quantitative result was within or close to the acceptable degree of variation of 0.5 log₁₀ copies/ml (Table 2).

EBV

The qualitative and quantitative performance of EBV viral load testing was also performed using the two sample panels: a commercial preparation and the WHO International EBV Standard. As shown in Table 3, there were no false positive results reported on the negative control samples for either panel. There were discrepant results for the samples from the commercial panel expected to contain 3 or 4 log₁₀ copies/ml (1000 or 10,000 copies/ml). At these concentrations, no virus was detected using Protocol 5, while the other protocols were able to detect but not necessarily quantitate virus. Quantitative results were reported from all five protocols for the commercially prepared samples expected to contain at least 5 log₁₀ copies/ml (100,000 copies/ml). The mean EBV viral load on these samples was lower than the expected value at each of the concentrations tested (Table 3). The difference between the mean and the expected value at each concentration ranged between 0.71 and 0.96 log₁₀ copies/ml. The individual results from each protocol were also all less than the expected value, with 8 of the 20 samples falling within 0.5 log₁₀ copies/ml of the expected result (Figure 2). When compared, the difference between the highest result and the lowest result was more than 1.5 log₁₀ copies/ml at every concentration.

As shown in Table 3, for the WHO International EBV Standard panel, EBV was detected in the majority of the replicates expected to contain 2 log₁₀ copies/ml (100 copies/ml). All of the protocols were able to detect, but not necessarily quantitate, virus in the samples expected to contain 3 log₁₀ copies/ml (1000 copies/ml). For samples expected to contain at least 4 log₁₀ copies/ml (10,000 copies/ml), all five protocols gave quantifiable results. In these cases, the mean viral load was higher than the expected value, with the difference ranging from 0.16 to 0.44 log₁₀ copies/ml. However, when considering data from each laboratory separately, the results were distributed above and below the expected value with the results obtained using protocols 2, 3, 5, and the majority of replicates from protocol 2 above the expected value, and the results from protocol 4 all below the expected value. Of the 42 samples containing at least 4 log₁₀ copies/ml (10,000 copies/ml), 26 (62%) were within 0.5 log₁₀ copies/ml of the expected result. When compared to each other, the difference between the highest result and the lowest result were more than 1.0 log₁₀ copies/ml at each concentration.

Discussion

International guidelines recommend the use of viral load testing for the diagnosis and management of both CMV and EBV infections in organ transplant recipients (1,2,5,17,18). Additionally, these assays provide valuable data for the evaluation of new immunosuppressive regimens or antiviral therapies in clinical trials. The purpose of this study was to compare the current laboratory practices in CMV and EBV viral load testing performed at four large transplantation centers. Similar to previous studies (10,11), we found considerable differences among CMV and EBV viral load values when commercially available viral panels were tested. In all cases, the observed viral load was lower than the

expected viral load, suggesting that either the expected concentration of virus in the viral panels was not accurate, or the assays were under calibrated. However, the CMV and EBV viral loads were relatively accurate (± 0.5 log₁₀ copies/ml of the expected value) when compared to the WHO Standards. At the time this study was performed, the commercial reagents were not related to any official standard, whereas the assigned value for the concentration of the WHO standards was established in a large multisite study. This may explain why the assays appeared more accurate using WHO standards as the reference material.

Each component of the testing method contributes to observed variation in viral load results, including differences in the extraction method, amplification reagents, genes targeted, and calibrators used (19). In one study comparing EBV viral load results obtained using the same amplification system, viral loads were in close agreement when the same extraction method was used, but varied 2.3 fold when different extraction methods were used (9). Others have shown that automated extraction and commercially available amplification systems tend to perform better than laboratory developed “home brew” assays (10). However, even among commercial systems, considerable differences among viral load values have been reported (8,10). The present data suggest that the assays used for CMV viral load testing provide results within the expected range of variation for this type of assay and thus may be compared from one center to the next. Variation was greater for EBV assays; the basis for this variability is unclear. It is possible that EBV results are more sensitive to differences in extraction methods or in the amplification efficiency for each assay as has been observed by others (9, 11, 12). Laboratory proficiency may also contribute to variability (11). Controversy persists as to which blood compartment should be used to measure EBV viral loads; variability in clinical assays reflects various clinical conditions with differing proportions of encapsulated and free viral DNA. Given the variability in EBV assay data and the variability in levels of EBV associated with clinical disease, for individual patient management it may be most appropriate to use a single EBV assay performed at one laboratory.

The WHO International Standards facilitate the standardization of CMV and EBV viral load testing. These standards will allow the use of multiple clinical laboratories rather than mandating the use of a central or core laboratory in the performance of clinical trials (21). This observation assumes that participating laboratories will demonstrate proficiency in assay performance across the reportable ranges in advance of the trial. In this series, the lower limits of detection or sensitivity of the assays varied between sites. Data do not exist to define a clinically “important” lower limit for reportable viral loads for CMV and EBV. The assays are used in both detection and management of infection and, in practice, viral loads vary widely. While some clinical laboratories use plasma as the clinical specimen of choice and others use whole blood; viral load values cannot be compared across different specimen types given the presence of EBV and CMV DNA in peripheral blood cells that are not present in plasma. Each laboratory will need to convert viral load values from copies to international units. Commercial suppliers will need to recalibrate their assay platforms to the international standards. Laboratories using “home brew” platforms will require experimentation to determine appropriate conversion factors. The availability of viral standards should facilitate clinical trials in the future and also enhance the performance of

clinical laboratories for patient care. However, viral load assay standardization must be performed in advance of the initiation of multicenter trials.

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Abbreviations

CTOT	Clinical Trials in Organ Transplantation
CMV	Cytomegalovirus
EBV	Epstein-Barr virus
IE	Intermediate Early
qPCR	Real-Time quantitative polymerase chain reaction

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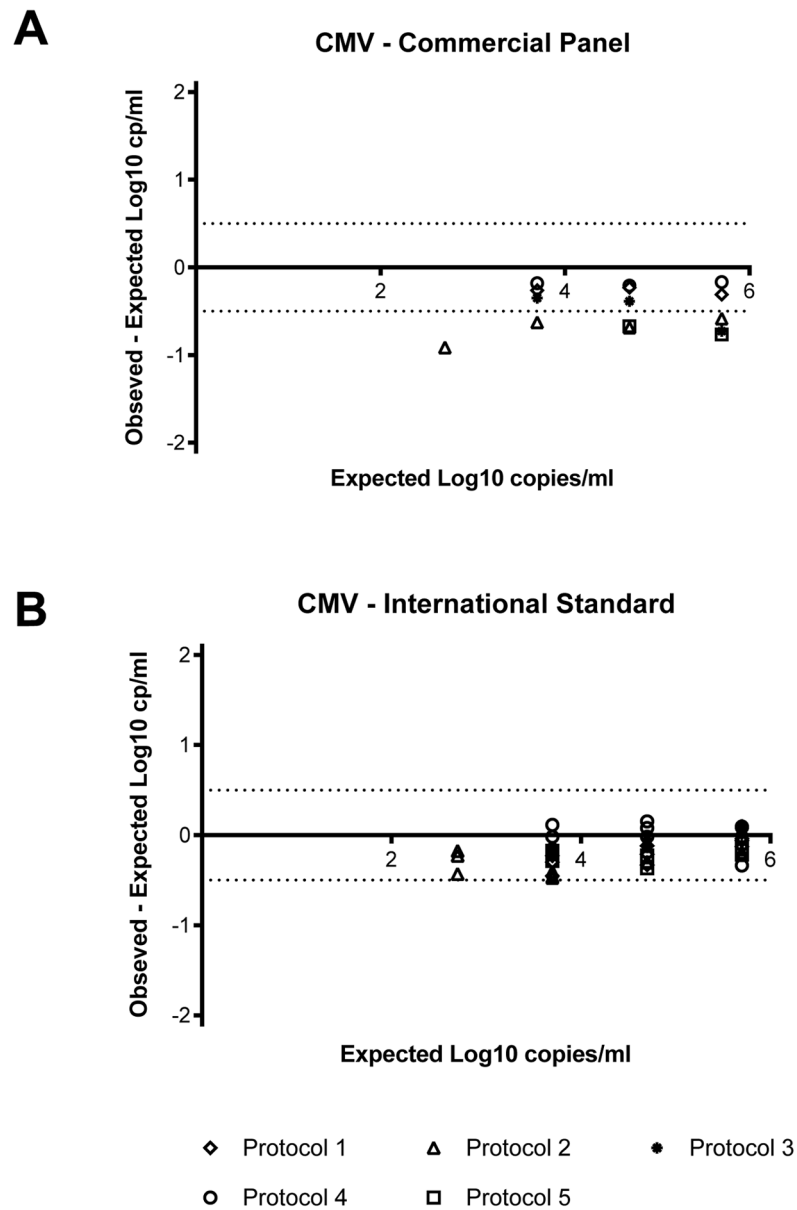


Figure 1. Accuracy of CMV viral loads

Each point represents an individual replicate from the protocol as indicated in the legend. Negative results and viral load values below the reportable range of the assay for each protocol are not included. The horizontal dotted lines correspond to the acceptable range of variation of $\pm 0.5 \log_{10}$ copies/ml from 0.

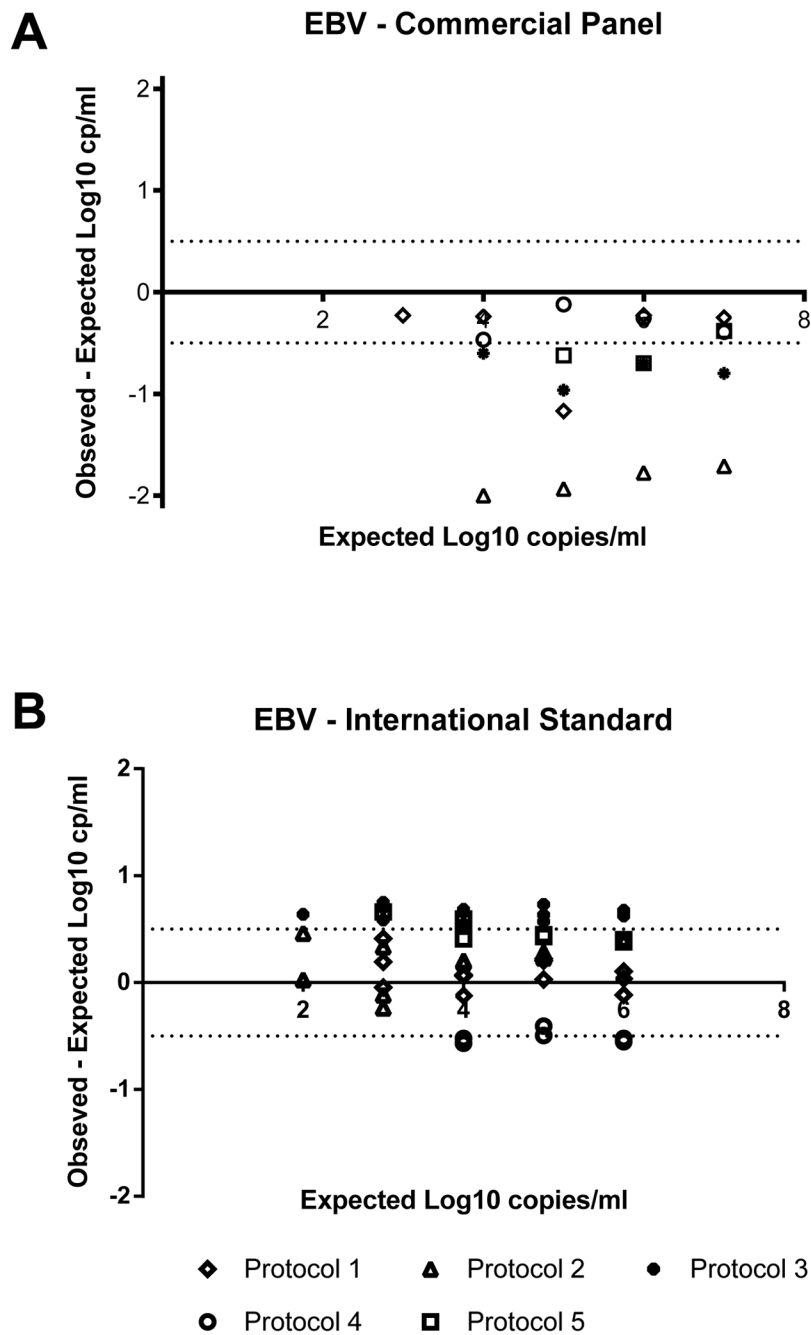


Figure 2. Accuracy of EBV viral loads

Each point represents an individual replicate from the protocol as indicated in the legend. Negative results and viral load values below the reportable range of the assay for each protocol are not included. The horizontal dotted lines correspond to the acceptable range of variation of $\pm 0.5 \log_{10}$ copies/ml from 0.

Table 1

Testing Protocols

	Nucleic Acid Extraction	Amplification and Detection	Target and Amplicon Size	Reportable Range
1	QiAmp Virus on Qiagen BioRobot MDX	Qiagen artus™ EBV/CMV on Applied Biosystems 7500 Real-Time PCR System	EBV EBNA1 Amplicon size: 97 bp CMV Major IE Amplicon size: 105 bp	EBV 500–5,000,000 cp/ml CMV 313 – 3,130,000 cp/ml
2	QiAmp DNA Blood Mini Kit on QiaCube	Qiagen artus™ EBV/CMV on Applied Biosystems QuantStudio 12K Flex	EBV EBNA1 Amplicon size: 97 bp CMV Major IE Amplicon size: 105 bp	EBV >25 cp/ml CMV >50 cp/ml
3	Qiagen Virus/Bacteria Mini/Midi kit on QiaSymphony	Qiagen artus™ EBV/CMV on Qiagen RotorGene Q	EBV EBNA1 Amplicon size: 97 bp CMV Major IE Amplicon size: 105 bp	EBV 300–1,500,000 cp/ml CMV 1000–5,000,000
4	Qiagen MinElute kit on QiaCube	Primera Dx ViraQuant on ICEPlex	EBV EBNA-LP Amplicon size: 155 bp CMV US28 Amplicon size: 171 bp	EBV 750–15,000,000 cp/ml CMV 750–15,000,000 cp/ml
5	MagNA Pure Compact Nucleic Acid Isolation Kit 1 on Roche MagNA Pure Compact	Lab developed assays on ABI 7500 Real-Time System (EBV) and ABI 7300 Real-Time PCR System (CMV)	EBNA1 Amplicon size: 68 bp CMV UL54 Amplicon size: 61 bp	EBV 4,000–40,000,000 cp/ml CMV 2,000 – 1,250,000 cp/ml

Table 2

Summary of Qualitative Performance for CMV Viral Load Testing

CMV – Commercial panel - Single replicate of each dilution at each site						
Expected copies/ml (log ₁₀ copies/ml)	Negative	Positive*	Quantifiable	Mean (log ₁₀ copies/ml)	Range (log ₁₀ copies/ml)	
0	5					
500 (2.7)	1	4				
5000 (3.7)		1	4	3.34	3.07–3.52	
50,000 (4.7)			5	4.26	4.01–4.49	
500,000 (5.7)			5	5.19	4.94–5.53	
CMV – WHO International Standard, three replicates of each dilution at each site						
Expected IU/ml (log ₁₀ IU/ml)	Negative	Positive*	Quantifiable	Mean (log ₁₀ copies/ml)	Range (log ₁₀ copies/ml)	
0	15					
5 (0.7)	13	2				
50 (1.7)	10	5				
500 (2.7)		12	3	2.42	2.27–2.52	
5000 (3.7)		1	14	3.46	3.23–3.81	
50,000 (4.7)			15	4.54	4.33–4.85	
500,000 (5.7)			15	5.61	5.36–5.81	

* Positive but below the reportable range

Table 3

Summary of Qualitative Performance for EBV Viral Load Testing

EBV – Commercial panel - Single replicate of each dilution at each site						
Expected (log ₁₀ copies/ml)	Number of replicates			Mean (log ₁₀ copies/ml)	Range (log ₁₀ copies/ml)	
	Negative	Positive *	Quantifiable			
0	5					
3	1	3	1	2.27		
4	1		4	3.17	2.00–3.76	
5			5	4.04	3.07–4.88	
6			5	5.27	4.23–5.77	
7			5	6.29	5.29–6.75	
EBV – WHO International Standard, Three replicates of each dilution at each site **						
Expected IU/ml (log ₁₀ IU/ml)	Number of replicates			Mean (log ₁₀ copies/ml)	Range (log ₁₀ copies/ml)	
	Negative	Positive *	Quantifiable			
0	14					
10 (1)	10	4				
100 (2)	4	7	3	2.44	2.01–3.16	
1000 (3)		4	10	3.38	2.37–3.75	
10,000 (4)			14	4.16	3.45–4.69	
100,000 (5)			14	5.21	4.55–5.73	
1,000,000 (6)			14	6.18	5.46–6.67	

** Protocol 4 only 2 replicates;

* Positive but below the reportable range