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Validation of the CMVPCR/CMV QNT using the QuantStudio 12K Flex Real-Time PCR System

By Shuangyun Lu

A thesis

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Committee Approval

To the Graduate Faculty:

The members of the committee appointed to examine the thesis of

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I want to dedicate this paper to my family and my parents for their continuous support and encouragement.

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LIST OF ABBREVIATIONS

PCR	Polymerase chain reaction
CMV	Cytomegalovirus
CEP	Cytopathic effect
ELISA	Enzyme-linked immunosorbent assay
BAL	Bronchoalveolar lavage
CSF	Cerebrospinal fluid
DBS	Dried blood spot
LOD	Limit of detection
CT	Threshold cycle
CV	Coefficient of variance
IC	Internal control

ABSTRACT

Polymerase chain reaction (PCR) methods are extensively used in diverse clinical laboratories due to high reliability, high sensitivity and productivity. The purpose of this validation is to transit the related cytomegalovirus (CMV) by qualitative PCR and CMV DNA quantitation by PCR assays from the ABI Prism 7900HT (ABI7900) using the Promega Protocol to the QuantStudio™ 12K Flex Real-Time PCR System (QuantStudio) using Protocol 1. Limit of detections (LODs) of CMV in whole blood, plasma, serum, CSF, BAL, bone marrow, DBS, ocular fluid, saliva and urine were the same on both QuantStudio and ABI7900. In amniotic fluid, QuantStudio had a lower LOD than the ABI7900. The accuracy of QuantStudio had a 100% agreement with the ABI7900. The intra-run precision and inter-run precision were also comparable and less than 5% on both analyzers. Therefore, CMVPCR and CMV quantitation assays can be successfully performed using the QuantStudio.

Validation of the CMVPCR/CMV QNT

Using the QuantStudio 12K Flex Real-Time PCR System

1. Introduction

Cytomegalovirus (CMV) is one of the herpesviruses family and is found worldwide. The characteristic of this family is the ability to establish latent infections with sporadic episodes of reactivation of disease or viral shedding. In the United States, 80% of people will be infected with CMV at some point. Most infections are asymptomatic in healthy individuals; however it can cause substantial morbidity and mortality in immunocompromised patients, and hearing loss or developmental disabilities in congenital CMV infection children. In the United States, 1 in 150 children are born with congenital CMV infection, and 20% of those will develop permanent problems. CMV is also the major viral pathogen following organ transplantation. During active CMV infection, the virus disseminates in the blood, which presents a significant risk factor for development of end-organ disease, such as pneumonitis, retinitis, gastritis, enteritis, CNS disease, nephritis, etc. Therefore, early detection of CMV infection is essential. During the past decade, major advances have been achieved in the diagnostic techniques for the rapid identification of CMV virus, such as quantification of CMV viral load through PCR. However, conventional methods such as cell culture and serological tests are still commonly used.

Cell culture detects the presence of virus by the recognition of characteristic cytopathic effect (CPE) in human fibroblast cell cultures (MRC-5 cells). CPE is directly related to a virus's titer. Viral culture can be performed on various sample types, such as blood, respiratory secretions, saliva, urine, stool, CSF and tissue biopsy specimens. Cell culture usually requires 2-21 days for CPE to occur and sometimes even longer, which limits its utility in contemporary clinical practice. Shell vial assay, also called the CMV rapid test, is a modified viral culture method. In the shell vial assay, the patient sample is centrifuged onto a single layer of human diploid fibroblast cells (MCC-5) and viral growth is measured by antigen detection methods, which

greatly reduces the turnaround time and enhances the sensitivity. It is markedly less sensitive than the conventional cell culture [1], but it makes screening for the large volume possible by using a 96-well plate [2].

Serological tests detect the presence or absence of CMV antibodies (IgG or IgM) in serum, which is a primary method to assess the potential for CMV infection. Acute infection is typically characterized by increased CMV-specific IgM and IgG antibodies. Many different assays have been described and evaluated, such as enzyme-linked immunosorbent assay (ELISA), complement fixation, anti-complement immuno-fluorescence and radio-immunoassay. Among these, ELISA is the most commonly used. Traditionally, CMV-IgG antibodies have been used to assess whether a patient had CMV infection in the past. CMV-IgM is a sensitive marker for acute or recent infection. However, IgM antibodies can also be produced during CMV reactivation, and remain detectable for unpredictable lengths of time. These factors make IgM a poor diagnostic tool in primary CMV infection during pregnancy. As a result, CMV IgG avidity has become the recommended method to determine the risk of a primary CMV infection during the first trimester of pregnancy [3]. Low avidity index indicates primary infection within the preceding 3-4 months and high avidity index indicates past CMV infection/reactivation [4]. However, CMV serology assays are not recommended for diagnosis in immunocompromised patients because CMV serology (IgG or IgM antibody titers) may not be reliable and therefore misleading in the diagnosis of acute or reactivation CMV disease in patients. The preferred method for these aforementioned patients is demonstration of viral antigen.

An Antigenemia assay, which detects the viral pp65 antigen, is a commonly used method for CMV virus quantification in blood specimens. Pp65 antigen is secreted by CMV-infected leukocytes during the early phase of the CMV replication process, so its detection in peripheral blood generally indicates active CMV infection [5]. After comparing antigenemia assays, PCR, serology and shell vial assays, Kazunari found that antigenemia assays have good correlation with the clinical course and the results of the assay in transplant recipients [6]. Antigenemia is rapid and sensitive for patients with CMV disease who have a higher viral load. However, it is labor intensive with low throughput and it may not be used for patients with no symptoms

and patients with severe leukopenia (define- low white count). In addition, those samples have to be processed rapidly because test results depend on the life span of leukocytes, which may be less than 8 hours.

An Immunohistochemistry method is performed primarily on tissue or body fluid samples. Fluorescent or light microscopy is used to examine the color change of the substrate. This technique is sensitive and very specific. However, immunohistochemistry detection of CMV infection is very labor intensive and is subjective, requiring experienced personnel to read the slides. Therefore, it is often used as a confirmatory test.

During the past two decades, PCR and several other DNA/RNA amplification techniques have become important diagnostic tools in clinical laboratories. The development of real-time PCR, has revolutionized the way clinical laboratories diagnose many human infectious diseases because it allows users to monitor the reaction as it progresses. Real-time PCR can be used quantitatively, semi-quantitatively. Quantification of CMV viral load in peripheral blood leukocytes or plasma has been shown to correlate with progression of disease, particularly in bone marrow transplant recipients. Measurement of viral load also provides a potential mechanism for monitoring response to therapy because rise in viral load over time is more important in predicting CMV disease than a single viral load result at a given time point. CMV by PCR is a more sensitive method for the detection of CMV viremia and central nervous system infections, especially in the immunocompromised patients. Detection of CMV in saliva by qualitative PCR is potentially useful for congenital screening of neonates for CMV-associated hearing loss [A]. PCR methodology is generally more expensive compared to the antigenemiaassay, but it has fast turnaround time and extremely high sensitivity. Due to its specificity, sensitivity and potential for speed, PCR is accepted as the diagnostic gold standard for the detection of many viruses, including CMV.

The ABI7900 and Quantstudio™ are both the real-time PCR system and they are both used in the ARUP diagnostic labs. However, Quantstudio™ is very stable and has friendly interface. It takes two and half hours to finish the amplication process in

ABI 7900, where it only takes one and half hours in Quantstudio™, resulting in faster turnaround times.

The purpose of this project is to transition the related cytomegalovirus by PCR assays from the ABI Prism 7900HT (ABI7900) using the Promega Protocol currently in use at ARUP (Associated Regional and University Pathologists Laboratory, Salt Lake City, UT) to the QuantStudio™ 12K Flex Real-Time PCR System (QuantStudio) using Protocol 1. Any time an assay is moved from one instrument to another, or a different technology is used, a validation study must be done to conform to CLIA 88, the law which governs the operation of Medical Laboratories. This validation includes analytical sensitivity data in the form of a limit of detection (LOD) study for CMV in any matrix submitted for analysis, including amniotic fluid, BAL, bone marrow, CSF, dried blood spot (DBS), ocular fluid, plasma, saliva in ORACollect media, serum, urine, and whole blood. It must also include analysis for accuracy and precision and coefficient of correlation (comparison) to existing standard methodology. Although relatively simple for assays reported as numerical results, it is problematic in positive/negative determinations where cutoff values must be determined. The form of the study is left to the individual lab to develop if the methodology is recognized by the FDA and has gone through the rigorous vetting process conducted when the assay is approved.

2. Materials and Methods

In this study Calibrator material was prepared and standard curves generated using both the QuantStudio™ and ABI7900 instruments (currently in use and newly introduced analyzers). The same calibrator material was used for both analyzers. Accuracy was assessed using previously tested CMVPCR samples, 22 of which previously tested positive for CMV and 14 of which tested as CMV negative. Samples are de-identified and are part of the sample bank maintained by ARUP Laboratories. Intra-and inter-run precision was compared between the QuantStudio™ and ABI7900.

Instruments

ABI Prism 7900HT (ABI7900), QuantStudio™ 12K Flex Real-Time PCR System (QuantStudio) were used for the analysis. Chemagic Magnetic Separation Module I (PerkinElmer), Hamilton (Microlab star^{let}), Viaflo (Integr), Maxwell (Promega MDX), Microcentrifuge, Vortex, and a Plate Centrifuge were used in the preparation of the samples for analysis.

Reagents

Relevent reagents used were AcroMetrix^{TM} CMV_{TC} Panel (Thermo Scientific), CMV ASR detection reagent (Elitech /Epoch Biosciences), Uracil N-Glycosylase (UNG) (APplied Biosystems), ARUP Hot Start MasterMix (Promega, Madison, WI USA), $MgCl_2$ Solution (Promega, Madison, WI USA), PBS, molecular grade water (Mediatech, Inc), Chemagic kit (PerkinElmer). Reagent preparation and setting was made according to manufacturer's instructions.

Samples

In this study Calibrator material was prepared and standard curves generated using both the QuantStudio™ and ABI7900 instruments (currently in use and newly introduced analyzers). Accuracy was assessed using previously tested CMVPCR samples, 22 of which previously tested positive for CMV and 14 of which tested as

CMV negative. Intra- and inter-run precision was compared between the QuantStudio™ and ABI7900.

The test samples consisted of random samples sent to ARUP laboratories and maintained frozen. All samples were handled at all times according to standard precautions.

Analytical Sensitivity

5-fold dilution series of quantified, whole CMV were made in CMVPCR negative sample pools of amniotic fluid, BAL, bone marrow, CSF, DBS, ocular fluid, plasma, saliva in ORACollect media, serum, urine, and whole blood. These dilution series were extracted in triplicate. Each extracted dilution series was then amplified in duplicate using the QuantStudio. For comparison this same LOD template was amplified using the ABI7900. These LODs were tested as a side by side comparison to demonstrate equivalent sensitivity between methods.

Standard Curve Comparisons

Standard curves were generated using a 5-member calibration panel from AcroMetrix consisting of intact human CMV (strain AD169) serial diluted (10-fold) in a normal human plasma matrix. This calibration material was extracted in duplicate using the Chemagic. The resulting template was pooled by dilution to create a uniform standard curve template with sufficient volume to amplify two separate standard curves. This template was amplified/detected using both the QuantStudio and the ABI7900. Each instrument's native software package was used to generate standard curve values. The standard curve establishes the linearity of the results over the anticipated range of results.

Accuracy

Accuracy for this assay was evaluated using previously tested CMVPCR (and related PCR tests) samples representing the validated sample types. Thirty-three previously tested samples were used. Twenty-two of these samples previously tested CMV

positive in the clinical lab. Eleven un-spiked CMV negative samples were also included.

Precision

Intra-Run Precision

Previously tested CMVPCR plasma samples which tested positive for CMV at varying levels were selected for precision studies. These samples were extracted and amplified in triplicate on the same run and then analyzed according to standard procedure using both the QuantStudio and ABI7900. Mean, standard deviation and coefficient of variation (CV) were calculated for each level of repeated measures. These calculations were made on the raw count numerical data.

Inter-Run Precision

Previously tested CMVPCR plasma samples which tested positive for CMV at varying levels were selected for precision studies. These samples were extracted and amplified in triplicate on three separate runs. Both the QuantStudio and ABI7900 were evaluated. Mean, standard deviation and CV were calculated for each level of repeated measures.

3. Results

3.1. CMVPCR/CMV QNT Analytical Sensitivity in whole blood

Dilution	Copies/mL	QuantStudio - CMV Ct						ABI7900 - CMV Ct		
		Rep A1	Rep B1	Rep C1	Rep A2	Rep B2	Rep C2	Rep A1	Rep B1	Rep C1
1	100,000	32.1	31.9	31.7	32.5	32.6	31.9	32.1	32.0	31.6
1:5	20,000	33.9	34.0	34.1	34.8	34.6	34.6	33.9	34.0	34.1
1:25	4,000	35.9	36.0	36.3	36.9	38.0	37.1	36.3	36.5	36.4
1:125	800	40.2	38.7	39.1	39.1	39.8	41.3	39.2	38.3	40.4
1:625	160	NEG	NEG	45.2	47.2	42.9	41.4	NEG	45.5	42.8
1:3125	32	43.7	NEG	NEG	43.0	NEG	NEG	45.6	NEG	45.7
1:15625	6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
1:78125	1	NEG	NEG	NEG						

Indicates limit of detection of CMV in Whole Blood using both the QuantStudio (Protocol 1) and ABI7900.

Table 1 CMVPCR/CMV QNT Analytical Sensitivity-CMV in whole blood

3.2. CMVPCR/CMV QNT Analytical Sensitivity in Plasma

Dilution	Copies/mL	QuantStudio - CMV Ct						ABI7900 - CMV Ct		
		Rep A1	Rep B1	Rep C1	Rep A2	Rep B2	Rep C2	Rep A1	Rep B1	Rep C1
1	100,000	32.7	32.8	32.7						
1:5	20,000	35.0	34.7	34.7	34.7	34.6	34.6	33.1	33.4	33.4
1:25	4,000	36.8	36.9	36.8	37.6	37.1	37.4	35.7	35.9	35.5
1:125	800	38.6	39.8	39.6	39.0	40.3	40.1	37.0	37.8	36.9
1:625	160	NEG	43.3	44.3	41.1	43.3	41.1	42.0	NEG	39.8
1:3125	32	NEG	NEG	43.7	43.6	NEG	NEG	40.8	NEG	39.9
1:15625	6	NEG	NEG	NEG	NEG	43.9	44.7	NEG	NEG	NEG
1:78125	1	NEG	NEG	NEG	NEG	NEG	NEG			

Indicates limit of detection of CMV in plasma using both the QuantStudio (Protocol 1) and ABI7900.

Table 2 CMVPCR/CMV QNT Analytical Sensitivity-CMV in plasma

3.3. CMVPCR/CMV QNT Analytical Sensitivity in Serum

Dilution	Copies/mL	QuantStudio - CMV Ct						ABI7900 - CMV Ct		
		Rep A1	Rep B1	Rep C1	Rep A2	Rep B2	Rep C2	Rep A1	Rep B1	Rep C1
1	100,000	32.7	32.3	32.7						
1:5	20,000	35.0	35.1	34.6	34.5	34.5	34.5	33.3	33.2	33.3
1:25	4,000	37.3	36.9	36.7	36.6	36.7	36.7	34.9	35.2	35.0
1:125	800	39.9	39.4	38.8	40.1	39.1	40.0	37.8	39.8	36.5
1:625	160	42.0	41.4	44.9	41.7	NEG	42.2	40.2	40.9	NEG
1:3125	32	43.6	44.1	44.7	44.4	NEG	NEG	40.9	NEG	NEG
1:15625	6	NEG	NEG	NEG	NEG	NEG	NEG	41.1	NEG	39.8
Indicates limit of detection of CMV in serum using both the QuantStudio (Protocol 1) and ABI7900.										

Table 3 CMVPCR/CMV QNT Analytical Sensitivity-CMV in serum

3.4. CMVPCR/CMV QNT Analytical Sensitivity in CSF

Dilution	Copies/mL	QuantStudio - CMV Ct						ABI7900 - CMV Ct					
		Rep A1	Rep B1	Rep C1	Rep A2	Rep B2	Rep C2	Rep A1	Rep B1	Rep C1	Rep A2	Rep B2	Rep C2
1	100,000												
1:5	20,000	33.8	33.9	33.8	35.9	36.0	36.0	34.5	34.4	34.7	36.5	36.8	36.2
1:25	4,000	36.6	36.6	37.1	38.0	38.3	39.0	36.9	37.0	37.2	39.0	38.8	38.9
1:125	800	38.8	38.8	38.4	41.9	40.5	41.0	39.0	41.5	40.7	41.1	41.6	44.0
1:625	160	40.7	42.1	NEG	44.8	44.9	41.8	44.4	44.5	42.9	47.0	44.9	NEG
1:3125	32	41.1	NEG	42.7	NEG	NEG	NEG	43.8	NEG	NEG	NEG	NEG	NEG
1:15625	6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	46.3
Indicates limit of detection of CMV in CSF using both the QuantStudio (Protocol 1) and ABI7900.													

Table 4 CMVPCR/CMV QNT Analytical Sensitivity-CMV in CSF

3.5. CMVPCR/CMV QNT Analytical Sensitivity in BAL

Dilution	Copies/mL	QuantStudio - CMV Ct						ABI7900 - CMV Ct		
		Rep A1	Rep B1	Rep C1	Rep A2	Rep B2	Rep C2	Rep A1	Rep B1	Rep C1
1	100,000	32.1	31.9	31.7	32.6	32.5	32.3	30.9	31.2	31.2
1:5	20,000	34.2	33.8	34.2	34.6	34.5	34.7	33.6	33.3	33.4
1:25	4,000	36.4	36.5	37.1	36.9	37.0	38.4	35.5	35.3	36.2
1:125	800	40.1	38.9	42.0	39.9	39.3	39.0	37.8	37.7	37.8
1:625	160	42.8	44.6	40.6	NEG	42.4	42.3	NEG	41.5	43.1
1:3125	32	NEG	41.8	NEG	NEG	NEG	NEG	NEG	NEG	NEG
1:15625	6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
Indicates limit of detection of CMV in BAL using both the QuantStudio (Protocol 1) and ABI7900.										

Table 5 CMVPCR/CMV QNT Analytical Sensitivity-CMV in BAL

3.6. CMVPCR/CMV QNT Analytical Sensitivity in Amniotic Fluid

Dilution	Copies/mL	QuantStudio - CMV Ct						ABI7900 - CMV Ct		
		Rep A1	Rep B1	Rep C1	Rep A2	Rep B2	Rep C2	Rep A1	Rep B1	Rep C1
1	100,000	33.3	33.5	32.9						
1:5	20,000	35.2	35.4	35.0	34.4	35.3	35.3	33.1	33.2	33.5
1:25	4,000	37.8	37.3	37.8	37.5	37.7	36.8	35.1	34.9	36.0
1:125	800	40.5	43.0	40.1	38.9	40.4	41.3	38.0	38.3	39.0
1:625	160	44.7	42.3	43.8	42.1	44.2	45.6	NEG	NEG	39.1
1:3125	32	NEG	NEG	NEG	44.7	44.6	NEG	39.3	NEG	NEG
1:15625	6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
Indicates limit of detection of CMV in amniotic fluid using the QuantStudio (Protocol 1).										
Indicates limit of detection of CMV in amniotic fluid using the ABI7900.										

Table 6 CMVPCR/CMV QNT Analytical Sensitivity-CMV in Amniotic Fluid

3.7. CMVPCR/CMV QNT Analytical Sensitivity in Bone Marrow

Dilution	Copies/mL	QuantStudio - CMV Ct						ABI7900 - CMV Ct					
		Rep A1	Rep B1	Rep C1	Rep A2	Rep B2	Rep C2	Rep A1	Rep B1	Rep C1	Rep A2	Rep B2	Rep C2
1	100,000	33.4	32.7	33.0	34.4	33.6	33.5	32.5	32.5	32.3	31.7	31.7	31.2
1:5	20,000	35.1	35.1	35.3	35.2	35.9	36.2	34.5	34.3	34.2	33.9	33.9	33.9
1:25	4,000	37.7	37.2	38.0	37.8	38.0	37.5	36.3	35.9	36.3	35.5	36.0	36.5
1:125	800	39.9	40.5	41.5	40.4	42.6	41.1	38.5	40.9	39.9	37.1	38.9	37.4
1:625	160	NEG	46.3	NEG	42.9	NEG	NEG	40.5	40.7	41.1	42.4	41.4	NEG
1:3125	32	NEG	NEG	NEG	NEG	NEG	NEG	41.1	NEG	NEG	NEG	NEG	NEG
1:15625	6	NEG	NEG	49.5	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
1:78125	1	NEG	NEG	NEG	49.4	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
Indicates limit of detection of CMV in bone marrow using both the QuantStudio (Protocol 1) and ABI7900.													

Table 7 CMVPCR/CMV QNT Analytical Sensitivity-CMV in Bone Marrow

3.8. CMVPCR/CMV QNT Analytical Sensitivity in DBS (Maxwell)

Dilution	Copies/mL	QuantStudio - CMV Ct						ABI7900 - CMV Ct		
		Rep A1	Rep B1	Rep C1	Rep A2	Rep B2	Rep C2	Rep A1	Rep B1	Rep C1
1	100,000	37.6	36.4	38.3	38.0	37.3	38.1	36.6	35.9	36.9
1:5	20,000	41.6	39.8	40.0	40.7	40.6	41.0	38.3	38.7	39.2
1:25	4,000	NEG	42.1	41.1	41.6	43.9	46.7	NEG	NEG	42.3
1:125	800	NEG	NEG	41.7	NEG	NEG	NEG	NEG	NEG	NEG
1:625	160	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
1:3125	32	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
Indicates limit of detection of CMV in DBS (Maxwell) using both the QuantStudio (Protocol 1) and ABI7900. A single 3/16 inch diameter punch was extracted for each replicate.										

Table 8 CMVPCR/CMV QNT Analytical Sensitivity-CMV in DBS (Maxwell)

3.9. CMVPCR/CMV QNT Analytical Sensitivity in Ocular Fluid

Dilution	Copies/mL	QuantStudio - CMV Ct						ABI7900 - CMV Ct		
		Rep A1	Rep B1	Rep C1	Rep A2	Rep B2	Rep C2	Rep A1	Rep B1	Rep C1
1	100,000									
1:5	20,000	33.8	33.8	33.7	35.9	35.4	35.4	34.4	33.9	34.3
1:25	4,000	35.9	35.8	35.6	37.4	37.6	38.1	36.7	37.3	36.6
1:125	800	38.8	38.6	39.0	40.2	41.6	41.4	38.4	39.3	38.8
1:625	160	41.9	40.9	41.3	43.7	44.9	44.9	45.7	40.4	44.9
1:3125	32	NEG	NEG	42.6	45.2	NEG	NEG	NEG	44.3	NEG
1:15625	6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
Indicates limit of detection of CMV in ocular fluid using both the QuantStudio (Protocol 1) and ABI7900.										

Table 9 CMVPCR/CMV QNT Analytical Sensitivity-CMV in Ocular Fluid

3.10. CMVPCR/CMV QNT Analytical Sensitivity in Saliva in ORACollect

Dilution	Copies/mL	QuantStudio - CMV Ct						ABI7900 - CMV Ct		
		Rep A1	Rep B1	Rep C1	Rep A2	Rep B2	Rep C2	Rep A1	Rep B1	Rep C1
1	100,000	32.0	32.4	32.2	32.0	31.8	31.7	31.6	31.4	31.4
1:5	20,000	34.3	34.0	34.0	34.0	33.7	34.4	33.5	33.4	33.8
1:25	4,000	36.2	35.3	36.7	35.9	36.0	36.6	36.2	36.0	36.5
1:125	800	39.3	41.1	40.4	40.9	39.3	39.6	39.4	39.5	39.5
1:625	160	42.3	40.5	NEG	44.7	41.8	41.0	NEG	39.3	39.2
1:3125	32	NEG	NEG	41.2	NEG	NEG	44.0	NEG	NEG	NEG
1:15625	6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
1:78125	1	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
Indicates limit of detection of CMV in saliva in ORACollect using both the QuantStudio (Protocol 1) and ABI7900. These were extracted using the Maxwell vTNA extraction.										

Table 10 CMVPCR/CMV QNT Analytical Sensitivity-CMV in ORACollect

3.11. CMVPCR/CMV QNT Analytical Sensitivity in urine

Dilution	Copies/mL	QuantStudio - CMV Ct						ABI7900 - CMV Ct					
		Rep A1	Rep B1	Rep C1	Rep A2	Rep B2	Rep C2	Rep A1	Rep B1	Rep C1	Rep A2	Rep B2	Rep C2
1	100,000	36.4	36.7	36.0				36.4	36.4	36.0			
1:5	20,000	38.7	39.8	38.7	39.4	38.7	39.4	39.7	39.7	40.5	36.7	36.4	36.4
1:25	4,000	41.1	41.9	40.8	44.4	41.6	43.4	48.1	41.6	40.3	42.1	39.4	43.8
1:125	800	47.6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	45.4	40.9	42.2	43.1
1:625	160	NEG	47.6	NEG	NEG	NEG	44.6	NEG	NEG	44.6	NEG	NEG	NEG
1:3125	32	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
1:15625	6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
Indicates limit of detection of CMV in urine using both the QuantStudio (Protocol 1) and ABI7900.													

Table 11 CMVPCR/CMV QNT Analytical Sensitivity-CMV in urine

3.12. Standard Curve Comparison

Standard Curve Comparison		
cps/mL	**QuantStudio Ct**	**ABI7900 Ct**
3.9E6	26.9	27.1
3.9E5	30.5	30.6
3.9E4	33.5	33.6
3.9E3 mean	37.3	37.6
3.9E3 rep 1	37.0	37.4
3.9E3 rep 2	37.5	37.7
390 mean	41.7	43.3
390 rep 1	42.8	43.0
390 rep 2	41.5	42.5
390 rep 3	40.8	43.1
390 rep 4	NEG	44.6

Curve characteristics		
	QuantStudio	**ABI7900**
slope	-3.702	-3.730
Y-intercept	50.994	51.335
R^2 value	0.984	0.990

Table 12 CMVPCR/CMV QNT Analytical Standard Curve Comparison

3.13. Accuracy

CMV Accuracy - Previously Tested Clinical Samples										
ID	Source	CMV Qualitative Clinical Result	Clinical ABI7900 FAM (CMV) Ct	QuantStudio FAM (CMV) Ct	ABI7900 FAM (CMV) Ct	QuantStudio cps/mL	ABI7900 cps/mL	QuantStudio log copies/mL	ABI7900 log copies/mL	Δ log copies/mL
CMV ACC01	URINE†	POS	34.0	41.8	38.7	304	2,442	2.5	3.4	0.9
CMV ACC02	AMNIOTIC†	POS	28.9	28.5	28.4	1,191,722	1,410,740	6.1	6.1	0.1
CMV ACC03	WHOLE BLOOD	POS	32.5	35.3	35.5	17,351	17,611	4.2	4.2	0
CMV ACC04	BAL	POS	37.1	39.7	38.9	1,124	2,159	3.1	3.3	0.3
CMV ACC05	PLASMA	POS	31.5	31.5	32.0	184,418	152,830	5.3	5.2	0.1
CMV ACC06	PLASMA	POS	33.8	34.4	35.2	30,370	21,194	4.5	4.3	0.2
CMV ACC07	PLASMA	POS	29.8	29.4	30.0	680,868	525,354	5.8	5.7	0.1
CMV ACC08	BAL	POS	31.6	32.3	32.7	112,125	99,202	5.0	5.0	0.1
CMV ACC09	AMNIOTIC†	POS	29.4	28.2	29.0	1,436,191	974,033	6.2	6.0	0.2
CMV ACC10	SERUM†	POS	41.8	40.3	41.6	774	408	2.9	2.6	0.3
CMV ACC11	BONE MARROW†	POS	35.4	35.5	35.9	15,322	13,757	4.2	4.1	0.0
CMV ACC12	BRONCH WASH	POS	30.6	34.6	35.5	26,817	17,611	4.4	4.2	0.2
CMV ACC13	WHOLE BLOOD	POS	39.2	40.3	39.8	774	1,238	2.9	3.1	0.2
CMV ACC14	BAL	POS	26.3	27.7	28.1	1,960,083	1,697,789	6.3	6.2	0.1
CMV ACC15	URINE†	POS	21.7	20.4	20.6	183,730,613	174,100,197	8.3	8.2	0.0
CMV ACC16	SERUM	POS	33.9	33.8	34.5	44,108	32,651	4.6	4.5	0.1
CMV ACC17	AMNIOTIC†	POS	23.5	23.0	23.4	36,463,509	30,907,041	7.6	7.5	0.1
CMV ACC18	CSF†	POS	35.4	35.0	35.8	20,911	14,633	4.3	4.2	0.2
CMV ACC19	SERUM†	POS	28.1	28.1	28.7	1,528,357	1,172,223	6.2	6.1	0.1
CMV ACC20	VITREOUS FLUID†	POS	27.5	26.2	26.9	4,982,655	3,561,477	6.7	6.6	0.1
CMV ACC21	AMNIOTIC†	POS	27.2	29.7	30.1	564,971	493,901	5.8	5.7	0.1
CMV ACC22	WHOLE BLOOD	POS	36.6	37.1	37.3	5,664	5,796	3.8	3.8	0.0
CMV ACC23	WHOLE BLOOD	NEG	NEG	NEG	NEG	N/A	N/A	N/A	N/A	N/A
CMV ACC24	WHOLE BLOOD	NEG	NEG	IC fail	IC fail	N/A	N/A	N/A	N/A	N/A
CMV ACC25	BAL	NEG	NEG	42.3	40.6	223	756	2.3	2.9	0.5
drCMV ACC25	BAL	NEG	NEG	NEG	NEG	N/A	N/A	N/A	N/A	N/A
CMV ACC26	BAL	NEG	NEG	NEG	NEG	N/A	N/A	N/A	N/A	N/A
CMV ACC27	URINE	NEG	NEG	NEG	NEG	N/A	N/A	N/A	N/A	N/A
CMV ACC28	URINE	NEG	NEG	NEG	NEG	N/A	N/A	N/A	N/A	N/A
CMV ACC29	SERUM	NEG	NEG	NEG	NEG	N/A	N/A	N/A	N/A	N/A
CMV ACC30	SERUM	NEG	NEG	NEG	NEG	N/A	N/A	N/A	N/A	N/A
CMV ACC31	CSF	NEG	NEG	NEG	NEG	N/A	N/A	N/A	N/A	N/A
CMV ACC32	CSF	NEG	NEG	NEG	NEG	N/A	N/A	N/A	N/A	N/A
CMV ACC33	PLASMA	NEG	NEG	NEG	NEG	N/A	N/A	N/A	N/A	N/A
† This source is currently not quantifiable. Quantification value provided is merely informational.										

Table 13 CMVPCR/CMV QNT Analytical Accuracy

3.14.

CMV Accuracy - Summary of ICs				
ID	Source	CMV Qualitative Result	QuantStudio IC (PY559) Ct	ABI7900 IC (PY559) Ct
CMV ACC01	URINE	POS	33.9	34.3
CMV ACC02	AMNIOTIC	POS	32.2	32.6
CMV ACC03	WHOLE BLOOD	POS	33.2	33.7
CMV ACC04	BAL	POS	33.0	34.0
CMV ACC05	PLASMA	POS	32.5	33.6
CMV ACC06	PLASMA	POS	32.9	33.6
CMV ACC07	PLASMA	POS	32.1	33.5
CMV ACC08	BAL	POS	32.9	34.1
CMV ACC09	AMNIOTIC	POS	31.6	33.1
CMV ACC10	SERUM	POS	33.2	34.6
CMV ACC11	BONE MARROW	POS	33.1	35.2
CMV ACC12	BRONCH WASH	POS	33.1	34.2
CMV ACC13	WHOLE BLOOD	POS	33.3	33.9
CMV ACC14	BAL	POS	31.7	33.0
CMV ACC15	URINE	POS	30.9	33.9
CMV ACC16	SERUM	POS	33.1	34.4
CMV ACC17	AMNIOTIC	POS	30.5	33.5
CMV ACC18	CSF	POS	33.6	34.6
CMV ACC19	SERUM	POS	31.8	33.3
CMV ACC20	VITREOUS FLUID	POS	31.2	33.6
CMV ACC21	AMNIOTIC	POS	32.3	33.1
CMV ACC22	WHOLE BLOOD	POS	32.6	34.4
CMV ACC23	WHOLE BLOOD	NEG	32.8	34.6
CMV ACC24	WHOLE BLOOD	NEG	40.6	39.3
CMV ACC25	BAL	POS	33.1	34.0
drCMV ACC25	BAL	NEG	34.3	33.6
CMV ACC26	BAL	NEG	33.4	34.4
CMV ACC27	URINE	NEG	33.5	34.7
CMV ACC28	URINE	NEG	33.2	34.6
CMV ACC29	SERUM	NEG	33.4	34.0
CMV ACC30	SERUM	NEG	33.4	34.8
CMV ACC31	CSF	NEG	33.1	34.5
CMV ACC32	CSF	NEG	33.3	34.5
CMV ACC33	PLASMA	NEG	33.4	34.3
		Mean IC Ct	33.0	34.2
		IC Ct SD	1.588	1.083
		IC Ct %CV	4.81%	3.17%
		Negatives only - Mean IC Ct	33.4	34.4
		Negatives only - Ct SD	0.382	0.359
		Negatives only - Ct %CV	1.15%	1.04%
Indicates IC failure (> 3SD from negative IC mean).				

Table 14 CMVPCR/CMV QNT Analytical Accuracy - Summary of ICs

3.15. Precision -intra run

Intra-Run Precision (QuantStudio FAM Ct - CMV)							
ID	calculated cps/mL†	Ct#1	Ct#2	Ct#3	Mean Ct	SD (Ct)	%CV (Ct)
High	170,000,000	20.5	21.2	21.1	20.9	0.379	1.809%
Mid	21,000	35.8	35.9	35.8	35.8	0.058	0.161%
Low	2,100	40.1	41.5	39.7	40.4	0.945	2.338%
† Approximate value calculated using the ABI7900 standard curve used in this validation.							

Table 15 Intra-Run Precision (QuantStudio FAM Ct - CMV)

Intra-Run Precision (ABI7900 FAM Ct - CMV)							
ID	calculated cps/mL†	Ct#1	Ct#2	Ct#3	Mean Ct	SD (Ct)	%CV (Ct)
High	170,000,000	20.6	21.4	21.2	21.1	0.416	1.976%
Mid	21,000	35.2	36.2	36.0	35.8	0.529	1.478%
Low	2,100	38.9	40.6	40.3	39.9	0.907	2.272%
† Approximate value calculated using the ABI7900 standard curve used in this validation.							

Table 16 Intra-Run Precision (ABI7900 FAM Ct - CMV)

3.16. Precision -inter run

Inter-Run Precision (QuantStudio FAM Ct - CMV)							
ID	calculated cps/mL†	Ct#1	Ct#2	Ct#3	Mean Ct	SD (Ct)	%CV (Ct)
High	170,000,000	20.5	21.1	21.1	20.9	0.346	1.657%
Mid	21,000	35.8	36.7	35.4	36.0	0.666	1.851%
Low	2,100	40.1	41.5	38.6	40.1	1.450	3.620%
† Approximate value calculated using the ABI7900 standard curve used in this validation.							

Table 17 Inter-Run Precision (QuantStudio FAM Ct - CMV)

Inter-Run Precision (ABI7900 FAM Ct - CMV)							
ID	calculated cps/mL†	Ct#1	Ct#2	Ct#3	Mean Ct	SD (Ct)	%CV (Ct)
High	170,000,000	20.6	20.6	21.3	20.8	0.404	1.940%
Mid	21,000	35.2	34.3	36.2	35.2	0.950	2.698%
Low	2,100	38.9	37.9	40.1	39.0	1.102	2.827%
† Approximate value calculated using the ABI7900 standard curve used in this validation.							

Table 18 Inter-Run Precision (ABI7900 FAM Ct - CMV)

LOD was defined as the lowest concentration when all samples showed positive results. As seen in tables 1-10, both the QuantStudio and ABI7900 produced an equivalent LOD in most of the sample types, except for amniotic fluid. The QuantStudio was able to consistently detect CMV in amniotic fluid at a 5-fold lower dilution than the ABI7900.

From Table 13, 11 negative samples were tested as negative in both QuantStudio™ and ABI7900. 22 CMV positive samples were tested as positive in both QuantStudio and ABI7900 platforms.

From Table 15, the intra-assay coefficient of variation (measure of imprecision) on QuantStudio were 1.8%, 0.161% and 2.338% at high, middle and low CMV concentrations, respectively. While the intra-assay coefficient of variation on ABI7900 were 1.98%, 1.48%, and 2.27% at three different CMV concentrations, respectively. From table 17, the inter-assay variation coefficients on QuantStudio were 1.65%, 1.85% and 3.62% at high, middle and low CMV concentrations, respectively. The inter-assay CV on ABI 7900 were 1.94%, 2.70% and 2.83%, respectively. The generally accepted CVs for analytical tests are less than 5%. Both CVs of the intra-run and the inter-run are less than 5% on QuantStudio.

4. Discussion

Among the various clinical laboratory diagnostic tests currently available to detect CMV infection, nucleic acid amplification tests, such as PCR, are the most sensitive and specific detection methods. In addition, quantification of CMV DNA levels in peripheral blood (ie. CMV viral load) is used routinely to determine when to initiate preemptive antiviral therapy, diagnose active CMV disease, and monitor response to antiviral therapy. An increasing volume of published clinical studies demonstrate the utility of real-time PCR for diagnosing microbial pathogens. The high sensitivity and high specificity, short turnaround times for result, along with the relative ease of performance combine to make real-time PCR an attractive replacement method for conventional culture and antigen-based assays. In real time PCR, the amount of DNA is measured after each cycle via fluorescent dyes that yield increasing fluorescent signal in direct proportion to the number of PCR product molecules generated. Over the past several years, real-time PCR has become the leading tool for the detection and quantification of DNA or RNA.

There are many commercially available real-time PCR instruments, such as the ABI Prism series, Quantstudio, the MyiQ and iCycler. For laboratories with large numbers of specimens, the ABI Prism series (7000,7300 and 7500), Quantstudio™, the MyiQ and iCycler, Mx4000, MX3000p, Chromo4, Opticon and Opticon 2 and SynChron may be particularly useful. The ABI and Quantstudio™ are both used in the ARUP diagnostic labs. However, Quantstudio™ is very stable and has friendly interface. It takes two and half hours to finish the amplication process in ABI 7900, where it only takes one and half hours in Quantstudio™, resulting in faster turnaround times. Therefore, we tried to transit CMV by PCR assays from the ABI Prism 7900HT (ABI7900) using the Promega Protocol to the QuantStudio™ 12K Flex Real-Time PCR System (QuantStudio) using Protocol 1. From this study, we found that the ABI7900 and QuantStudio™ have equal LOD in most sample types (whole blood, serum, plasma, CSF, BAL, bone marrow, DBS, ocular fluid, saliva, and urine). In amniotic fluid, QuantStudio™ had an even lower LOD than the ABI7900.

Accuracy is compared between the QuantStudio™ and ABI7900 data, with the original clinical results given for reference. To generate quantitative values for the accuracy samples tested here the QuantStudio™ Curve and the ABI7900 Curve from the Standard Curve Comparison were used. Thirty-three samples were extracted using the Chemagic and tested using the QuantStudio™ and ABI7900 platforms. From table 13, we can see 11 negative samples were tested as negative in both QuantStudio™ and ABI7900 platforms. 22 CMV positive samples were tested as positive in both QuantStudio and ABI7900 platforms. Therefore, the accuracy of QuantStudio™ has a 100% agreement with the ABI 7900. From table 13 and 14, CMV ACC24 produced an IC failure using both the QuantStudio and ABI7900. This is not considered a discordant value since both instruments produced an inhibited result. The IC Ct %CV of CMV negatives is near 1% using both the QuantStudio and ABI7900.

There are a number of factors can affect viral load results, including the specimen type, biologic properties of the virus, performance characteristics of the quantitative assay (eg, limit of detection, limits of quantification, linearity, and reproducibility), degree of immunosuppression, and intensity of antiviral therapy. Among those, the acceptable specimen type is the first thing to be considered as a laboratory scientist.

CMV infection is spread through direct exposure to infected body fluids (saliva, urine or others), blood transfusion and organ transplantations. These viral particles can infect different target cells, such as epithelial cells, endothelial cells and fibroblasts, and smooth muscle cells [8]. These become different sources of different assays. In the ARUP diagnostic labs, amniotic fluid, BAL, bone marrow, CSF, dried blood spot (DBS), ocular fluid, plasma, saliva in ORACollect media, serum, urine, and whole blood are acceptable samples for CMV without disclaimer. There are about 800 CMV clinical samples for qualitative PCR and quantitation PCR in one week in our lab. Among those samples, are plasma and serum, whole blood samples, CSF, BAL, and urine.

The collection process of blood (whole blood, serum and plasma) is relatively easy compared with that of bone marrow and CSF. Therefore, blood is most

commonly used clinical sample to diagnose CMV disease. Whole blood, serum and plasma are from the same blood compartment; however, each may have its own advantage. Serum is formed after a complex clotting process following venipuncture and is expected to contain DNases. Both serum and plasma represent cell-free genomic DNA or viral nucleic acid as a way to monitor various diseases. Whole blood is cell-enriched samples. Research found that human CMV is present predominantly in infected polymorphonuclear leukocytes, and less is found in peripheral blood mononuclear cells [9-12]. Some studies showed that whole blood and leukocytes are more sensitive for CMV DNA detection than plasma and serum. [6, 13-16]. Therefore, the use of whole blood for CMV DNA detection is advocated by many researchers due to its higher sensitivity. Between plasma and serum, plasma is more sensitive for CMV DNA detection than serum [17]. In this experiment, the QuantStudio™ and ABI7900 have the same LOD in serum, plasma and whole blood. This may be because these samples are not real clinical samples, which were made by spiking same amount of CMV into different matrixes (whole blood, serum and plasma). In real clinical samples, CMV migrates into mononuclear cells and hides in it, which may increase the sensitivity of PCR in cell enriched samples. As mentioned earlier, only 6.8% are whole blood and 75% are plasma in our lab laboratories, which may decrease the detection of CMV. In order to increase the sensitivity of CMV detection in our lab, more measures should be taken to educate clients about the best specimen. However for the purposes of this study, the ability to detect the added viruses was the same for both analyzers.

When patients are suspected to have encephalitis, meningitis, or polyradiculopathy, CSF analysis usually can provide valuable diagnostic information. CSF is a clear, colorless body fluid that surrounds and protects the brain and spinal cord. The changes of glucose, white blood cell count and protein of CSF usually indicate meningitis. However, it cannot tell whether it is due to viruses or bacteria, or which organism causes this. The detection of virus in CSF is highly suggestive of central nervous system disease. Researchers found that PCR is useful in the rapid diagnosis of CMV infection of CNS in immunocompromised patients [18-19] and

quantitation of CMV DNA can be useful in monitoring antiviral therapy [20]. In this study, the LOD in CSF in Quantstudio is 800 copyies/mL.

BAL is widely used both diagnostically and therapeutically. It removes non-adherent cells and lung lining fluid from the mucosal surface. However, the detection of CMV in BAL fluid may or may not indicate CMV pneumonia. In addition, BAL is not a homogenous sample, which make the result more difficult to interpret. CMV ACC25 is a BAL specimen that was reported as CMV negative. The initial validation extraction of this specimen tested positive using both the QuantStudio and ABI7900, with Cts of 42.3 and 40.6, respectively. Upon re-extraction both the QuantStudio and ABI7900 produced negative results. This set of results suggests a level of CMV in the sample that is too low to reliably detect, or a possible CMV contaminant in the initial validation extraction.

Amniotic fluid, bone marrow, ocular fluid, saliva, urine and tissue are also acceptable samples for CMVPCR test in our lab. These sample types are only a small portion of all samples in our lab. When CVM invades different organs or tissue, these samples can provide importance critical information. Research found that CMV detection in amniotic fluid is more sensitive in antenatal diagnosis after 21 weeks' gestation than before 21 weeks' gestation [21]. However, there was no correlation between the CMV viral load in amniotic fluid and the fetal and neonatal outcomes [22-23]. CMV can also be detected in urine, even though high concentration of urea may have an inhibitory effect on the PCR for CMV DNA [24]. However, urine and stool are generally not recommended as sample types for CMV disease diagnosis, because viral shedding may be detected in it and it is of minimal clinical significance for transplant patients [5]. From table 1, 2, 3 and 11, the LOD is 4,000copies/mL in urine using both Quantstudio^{TM} and ABI 7900 and the LOD is 800copies/mL in blood samples. From our data, the urine sample is less sensitive for CMV DNA detection than blood samples, which may also limit urine as a common source for CMV DNA detection by PCR. However, the clinical utility of CMV DNA testing in urine is mainly for detection of CMV for neonatal screening of congenital CMV infection and CMV D+/R- SOT recipients [25]. Saliva is also reliable for neonatal screening of congenital CMV infection [26-27] and it is more easily collected than urine

specimens. CMV can also be detected in bone marrow. Some studies showed that in immunosuppressed patients detection is easier in CD34+ cells from blood and bone marrow. In general, higher CMV viral loads are associated with tissue-invasive disease, and tissue is also an acceptable sample for CMV PCR assay. In general, higher CMV viral loads are associated with tissue-invasive disease, while lower levels are associated with asymptomatic infection. However, the viral load in the peripheral blood compartment may be low or not detectable in some cases of tissue invasive disease. Therefore, tissue is also an acceptable source for CMVPCR detection.

5. Conclusions

This validation study demonstrates that the CMVPCR, CMVPCR SAL, and CMV QNT assays which are currently being performed on the AB17900 can be successfully performed using the QuantStudio. Accuracy, analytical sensitivity, and precision are comparable between the QuantStudio and ABI7900 instruments. The CMVPCR, CMVPCR SAL, and CMV QNT assays are now validated to be tested on the QuantStudio using Protocol 1.

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