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TECHNICAL ADVANCE

Standard Reference Material 2366 for Measurement of Human Cytomegalovirus DNA

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Human cytomegalovirus (CMV), classified as human herpesvirus 5, is ubiquitous in human populations. Infection generally causes little illness in healthy individuals, but can cause life-threatening disease in those who are immunocompromised or in newborns through complications arising from congenital CMV infection. An important aspect in diagnosis and treatment is to track circulating viral load with molecular methods, particularly with quantitative PCR. Standardization is vital, because of interlaboratory variability (due in part to the variety of assays and calibrants). Toward that end, the U.S. National Institute of Standards and Technology produced a Standard Reference Material 2366 appropriate for establishing metrological traceability of assay calibrants. This standard is composed of CMV DNA (Towne$_{\Delta 147}$ bacterial artificial chromosome DNA). Regions of the CMV DNA that are commonly used as targets for PCR assays were sequenced. Digital PCR was used to quantify the DNA, with concentration expressed as copies per microliter. The materials were tested for homogeneity and stability. An interlaboratory study was conducted by Quality Control for Molecular Diagnostics (Glasgow, UK), in which one component of SRM 2366 was included for analysis by participants in a CMV external quality assessment and proficiency testing program. *(J Mol Diagn 2013, 15: 177—185; http://dx.doi.org/10.1016/j.jmoldx.2012.09.007)*

Human cytomegalovirus (CMV), or human herpesvirus 5, is a member of the Herpesviridae virus family, which includes other viruses affecting humans: herpes simplex (human herpesvirus 1 and 2), varicella zoster virus (human herpesvirus 3), Epstein—Barr virus (human herpesvirus 4), and human herpesvirus 6, 7, and 8. Herpesviruses share a common architecture, and the human herpesviruses have genomes that range in size from 125,000 to 230,000 bp, with CMV (230,000 bp) having the largest genome of any herpesvirus known to date.[1] A large proportion of the general population is infected, from childhood onward. The infection is largely asymptomatic and becomes latent.[2,3] CMV can, however, cause life-threatening disease in two situations: in newborns infected *in utero* by a mother with an active CMV infection (ie, congenital infection) and in immunocompromised individuals.[3,4] The latter category includes patients in whom the weakened immune system either is deliberately induced for preservation of transplanted organs or stem cells or is the result of diseases that attack immune system cells, such as HIV/AIDS. CMV infection is a very important complication of allogeneic stem cell transplantation, resulting in CMV disease such as interstitial pneumonia, hepatitis, or gastric enteritis.[5] Treatment options are available, but are cytotoxic and have adverse side effects.[6,7]

Quantitative real-time PCR (qPCR) assays of CMV DNA viral load in plasma or whole blood are currently the method of choice for monitoring CMV infection.[8—11] In addition, the detection and quantitation of CMV viral load in dried blood stain has been of increasing clinical interest, particularly in the retrospective diagnosis of congenital infection.[12,13] Furthermore, CMV viral load can be detected earlier by qPCR than by antigenemia assays or culture.[5,14] Viral load and changes in viral load over time are used to

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determine the initiation, duration, and level of treatment.[4] The viral load at initial detection and the rate of increase correlate with the risk of CMV disease.[15] Pre-emptive treatment is initiated at the first positive test for CMV, and monitoring may be performed even when both the patient and the transplant donor are seronegative.[4]

Wolff et al[16] showed a high level of interlaboratory variability in testing. This variability is due in part to the use of a wide variety of methods for nucleic acid extraction and for qPCR, as well as a lack of quality reference materials and standards. A similar variability in testing was seen with proficiency testing results for other herpesviruses for which quantitation is an important diagnostic parameter, including human herpesvirus 6, for which testing also lacks standardization.[17] Interlaboratory reproducibility can be improved if the same platforms and reagents are used.[18,19] Interlaboratory variability becomes even more of an issue when the testing is done over long periods of time and in different laboratories. To address such variability, the National Institute of Standards and Technology (NIST) has developed Standard Reference Material (SRM) 2366: Cytomegalovirus for DNA Measurements. SRM 2366 is a preparation of CMV genomic DNA that has been developed to promote standardization, providing a mechanism for traceability for laboratory-developed and commercial calibrants to a primary NIST standard.

Materials and Methods

DNA Material Production

By definition, the SRM must be a consistent material. Culture of laboratory strains of CMV can vary over time and can also result in mutations, production of as little as 1% infectious particles, and large numbers of dense bodies consisting of tegument protein.[3] In addition, because of the large size of the genome, sometimes only part (or even none) of the genome is packed into the viral capsid.[3] To achieve consistency in the CMV DNA sequence, SRM 2366 consists of pure Towne strain CMV DNA cloned into a bacterial artificial chromosome (BAC). The development of this BAC, known as Towne$_{\Delta 147}$, is described by Marchini et al[20] and by Wang et al.[21] The BAC was subsequently provided to NIST by Hua Zhu (New Jersey Medical School, Newark, NJ). The BAC DNA is a construct that contains coding for the propagation of the CMV sequence in *Escherichia coli* and is only a minor component (~10,000 bp) of the total CMV BAC DNA (230,000 bp), but it links the two ends of the CMV DNA and the construct is essentially a very large plasmid.

The CMV Towne$_{\Delta 147}$ BAC was propagated at NIST in EPI300 *E. coli* cells grown in Luria–Bertani medium[22] containing 50 μg/mL kanamycin and 12.5 μg/mL chloramphenicol. An overnight culture was added to 1000 to 1200 mL of prewarmed (37°C) fresh medium and culture continued for 30 minutes, then 0.01% (final concentration) L-arabinose was added and culturing was continued for 2 hours at which time the cells were harvested. The cell pellets were suspended and washed with cold salt–Tris–EDTA buffer (100 mmol/L NaCl, 10 mmol/L Tris base, 1 mmol/L EDTA, pH 8.0).

Three batches of cells were grown separately, and the DNA was purified using an alkaline lysis method to release the BAC DNA from the harvested bacterial cells.[22] The BAC DNA pellet was dissolved in Tris–EDTA buffer (10 mmol/L Tris base, 1 mmol/L EDTA, pH 8.0). The 260 nm/280 nm parameter ranged from 1.94 to 2.01 and the 260 nm/230 nm parameter ranged from 2.28 to 2.60. The three pools of DNA were combined and then precipitated using saturated ammonium acetate and ethanol. The DNA was resuspended in 0.2× Tris–EDTA buffer. Dilutions of the stock CMV DNA were made under a laminar flow hood using sterile Tris–EDTA buffer and sterile perfluoroalkoxy (PFA) plastic bottles. Each of the three components (A, B, and C) was packaged on a separate day under a laminar flow hood. CMV DNA (150 μL) was pipetted into sterile 0.5-mL screw-capped PFA tubes (Savillex, Minnetonka, MN). For purposes of subsequent analysis (including quantification, stability, and homogeneity studies), one box of 100 consecutive tubes of a component was considered to be one lot.

To estimate the amount of *E. coli* DNA present, qPCR was performed as described previously.[23] The master mix was pretreated before amplification with the restriction endonuclease BanII, which cuts the *E. coli* DNA to eliminate *E. coli* DNA in the target sequence for amplification.[24] The qPCR assay results showed that *E. coli* DNA content was <1% of the total DNA.

As a check for possible interference with PCR, the DNA sequence for *E. coli* was probed for strong homologies with CMV primers and probes from published qPCR assays. This was done using the online Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*). The longest contiguous oligo matches were commonly 11 to 13 bases long. The *in silico* testing indicated that none of the primers and probe combinations of 72 CMV qPCR assays had sufficient homology with *E. coli* DNA to give a false positive. This was tested experimentally with 19 primer sets on *E. coli* EPI300 and SRM 2366. None of the PCR assays detected *E. coli* DNA and all detected CMV DNA.

DNA Sequence Analysis

Before the DNA was sequenced, PCR amplification was performed to purify the sample and to amplify the signal. PCR amplicons were run on an agarose gel (FlashGel DNA recovery system; Lonza, Walkersville, MD) to optimize the annealing temperature for the primer pair and to verify that only one product per primer pair was amplified. If multiple fragments were present after optimization of PCR amplification, the primer set was discarded. The PCR amplicons were purified enzymatically with exonuclease I and shrimp alkaline phosphatase (ExoSAP) or with ExoSAP-IT (all

from Affymetrix, Santa Clara, CA) to remove dNTPs and primers.

The ExoSAP/ExoSAP-IT–purified product was used as the template in Sanger sequencing with a BigDye terminator kit version 3.1 (Life Technologies–Applied Biosystems, Foster City, CA). Primers were designed using the online tool Primer3 version 0.4.0 (*http://primer3.wi.mit.edu*, last accessed January 15, 2012).[25] Many pairs were mixed and matched to resolve bases. Primers were designed to amplify fragments between 300 bp and 800 bp. Fragments within a region of interest were designed to overlap to generate continuous sequence reads. The CMV Towne strain (GenBank accession no. AY315197.2) was used as a reference. Two reactions are run, one using the forward primer and the other using the reverse primer, allowing reading of the sequence in both directions on the DNA strand. The products of BigDye were purified using Performa dye terminator removal spin columns (Edge BioSystems, Gaithersburg, MD) to remove unincorporated fluorescent ddNTPs. The purified sequencing reactions were diluted into HiDi formamide (Life Technologies–Invitrogen, Carlsbad, CA) and run on a capillary electrophoresis genetic analyzer (ABI 3130*xl*; Life Technologies–Applied Biosystems) with an 80-cm capillary and POP-7 performance-optimized polymer. The raw data were imported into Sequencher version 4.9 sequence analysis software (Gene Codes, Ann Arbor, MI) for processing.

Quantification of CMV DNA Using dPCR

Quantification of the reference material in genome copies per volume was accomplished using digital PCR (dPCR). The dPCR reactions were run on a BioMark platform (Fluidigm, San Francisco, CA) using 12.765 digital array integrated fluidic circuits, in which each array consists of 12 panels with 765 reaction chambers (6 nL each), for a total of 9180 individual PCR reactions per array. The fluorescent signal for each reaction chamber was read after every cycle of amplification. The concentration of DNA molecules in the amplification reaction mix was adjusted so that the average number of copies per reaction chamber was approximately 1.5[26] (ie, 400 to 600 positive chambers out of a total 765 chambers/panel). The PCR assay of Sassenscheidt et al,[27] known as CP1, was used for all of the quantification. The gene target for this assay is DNA polymerase (*UL54*), and the amplicon size is 72 bp. The PCR reactions consisted of 1× TaqMan Fast universal master mix (Life Technologies–Invitrogen), 1× GE sample loading reagent (Fluidigm), and uracil *N*-glycosylase (AmpErase UNG; Life Technologies–Applied Biosystems), with primer and probe concentrations of 300 nmol/L. Critical dilutions, such as addition of target DNA to the reaction mix, were performed using an analytical balance. Thermal cycling as follows: 50°C for 2 minutes, 95°C for 2 minutes, and 60 cycles of 95°C for 15 seconds and 60°C for 1 minute, with fluorescence measured at the end of the 60°C step. The BioMark software applies Poisson statistics to the data to calculate the number of positive chambers containing >1 DNA target molecule and estimates the total number of targets per reaction.

Each of the three SRM 2366 components (A, B, and C) was separately quantified. Three tubes were randomly selected for each component, and the DNA copy number (copies/μL) for each tube was measured using three arrays (33 panels with the complete reaction, and 3 panels serving as no-template controls). Each panel served as a data point. Thus, for each SRM component, a total of 99 data points went into the quantification and the calculation of associated uncertainty.

Six different assays using published primers and probes (Table 1)[10,11,27,28,29,30] were run on the BioMark platform to

Table 1 Quantitative PCR Assays for CMV

Assay	Target gene	Amplicon size (bp)	Reference		Sequence
CP1	*UL54*	72	26	Fwd	5′-GGCCGTTACTGTCTGCAGGA-3′
				Rev	5′-GGCCTCGTAGTGAAAATTAATGGT-3′
				Probe	5′-CCGTATTGGTGCGCGATCTGTTCAA-3′
gBA	*UL55*	254	27	Fwd	5′-TACCCCTATCGCGTGTGTTC-3′
				Rev	5′-ATAGGAGGCGCCACGTATTC-3′
				Probe	5′-TTGCTGCCCAGCAGATAAGTGGTG-3′
MIE 1F/1B	*UL122*	427	28	Fwd	5′-GCACCATCCTCCTCTTCCT-3′
				Rev	5′-GGCCTCTGATAACCAAGCC-3′
				Probe	5′-CCTCCTGAGCACCCTCCTCCTCTTCC-3′
MIE B short	*UL122*	138	29	Fwd	5′-CCAAGCGGCCTCTGATAACCAA-3′
				Rev	5′-GGTCATCCACACTAGGAGAGCAGAC-3′
				Probe	5′-TGAAGGTCTTTGCCCAGTACATTCT-3′
IE	*UL122*	127	11	Fwd	5′-CAAGCGGCCTCTGATAACCA-3′
				Rev	5′-ACTAGGAGAGCAGACTCTCAGAGGAT-3′
				Probe	5′-TGCATGAAGGTCTTTGCCCAGTACATTCT-3′
MIE 10	*UL126*	86	10	Fwd	5′-CC**C**GTGCCCGCAGTTTTTATT-3′
				Rev	5′-ACCGGAGAAGAGCCCATGTC-3′
				Probe	5′-AACATA**G**CGTGGGATCTCCACGCGAAT-3′

Bold type highlights two bases that were changed to match the Towne genome sequence and used at NIST (C in the MIE 10 forward primer and G in the MIE 10 probe).

determine whether the assay type made a difference in the resulting quantification. All six assays were run on an array, with two panels for each assay. The experiment was performed six times, for a total of 12 data points per assay.

Calculation of Measurement Uncertainties

The values and uncertainties of the components A, B, and C were computed using a Gaussian random-effects model with the lot as the random effect.[31] This model estimates the usual repeatability uncertainty, as well as a component that accounts for the variability between lots (homogeneity). The estimates are not in analytical form, but are computed using the lme function (linear mixed-effects function)[32] of the R statistical language (*http://www.r-project.org*).[33]

The model for each component is $y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$, where μ is the component mean, α_i is the effect of the ith lot, and ε_{ij} is the random measurement error for the ith lot, jth replicate. The α_i are Gaussian random variables with zero mean and variance σ_α^2, and the ε_{ij} are Gaussian random variables with zero mean and variance σ_ε^2.

Stability and Homogeneity Testing

For stability studies, tubes from all three components were subjected to three different temperatures (22°C, 37°C, and 65°C) for 1, 2, or 4 weeks each. Control tubes were kept at 4°C. Three tubes were selected for each condition and were assayed in triplicate. The aliquots of DNA were given approximately 40 minutes to reach room temperature, and all of the stability test materials were assayed on the same day. The CP1 assay[27] was used in a 96-well plate format, and reactions were distributed randomly on the plate. Reactions consisted of 1× TaqMan universal master mix (Life Technologies−Invitrogen), primers (400 nmol/L), probe (250 nmol/L), and template DNA, with water added to bring the volume to 20 μL. Reactions were run on an ABI 7500 real-time PCR system (Life Technologies−Applied Biosystems) with thermal cycling as follows: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, with fluorescence measured at the end of the 60°C step. The C_T was analyzed using the autoCt and autobaseline options in the ABI 7500 system. For the three different sets of data (one for each temperature condition: 22°C, 37°C, and 65°C), the analysis applied to the stability study was as follows. To check the stability of the material at elevated temperature, the data were fitted using the model $y_{ijkl} = \mu_i + \alpha_k + \beta_i x_j + \varepsilon_{ijkl}$, where y_{ijkl} is the measurement for the lth tube ($l = 1, 2, 3$) of the kth component ($k = 1, 2, 3$) for the jth week ($j = 1, 2, 4$) at the ith temperature ($i = 22, 37, 65$). The α_k represents a random component effect [ie, $\alpha_k \sim N(0, \tau_\alpha^2)$, where τ_α is the uncertainty that represents between-component variability], and ε_{ijkl} represents the usual measurement error. The stability of the material at the ith temperature can be judged by the size of the slope parameter β_i over time in weeks x_j. This analysis fits a regression over time and then tests whether the slope is 0.

For homogeneity studies, three tubes were removed from each lot of each component (12 to 15 lots) and were assayed in triplicate using the same CP1 assay[27] and the same thermal cycling protocol as for the stability studies, but with the baseline set to 0.27 C_T.

Interlaboratory Study

Component B of the SRM was provided to Quality Control for Molecular Diagnostics (QCMD; Glasgow, UK), to be included in their 2010 CMV External Quality Assessment (EQA) program. When component B was packaged, an additional 300 vials were filled for the interlaboratory study. These vials were shipped to QCMD, where they were labeled and included with other QCMD test materials sent out to 233 participants in 35 countries. The QCMD materials provided to participants consisted of 10 lyophilized samples with different concentrations of CMV virus (AD169 strain) in plasma or in viral transfer medium. Participants rehydrated the QCMD materials with water, extracted the DNA, and ran their qualitative or qPCR assay. The NIST component B material came with instructions to directly add the CMV DNA to the assay of the participant, without extraction and without adding water. SRM 2366 is designed primarily to provide traceability to the International System of Units (SI) for laboratory calibrants, not as a run control. However, the opportunity to include a sample in an EQA program meant that assays would be performed and results submitted by nearly 200 laboratories around the world. There are many CMV qPCR assays in use, both commercial and laboratory-developed. The 2012 CMV EQA provided an important opportunity to see how SRM 2366 works with the current variety of CMV assays.

Results

SRM 2366 Material

The SRM 2366 consists of pure DNA from the Towne strain of CMV cloned into a BAC,[20,21] $Towne_{\Delta 147}$, and propagated in *E. coli*. A unit of the SRM consists of three component tubes, each with the same DNA preparation, but at a different concentration. Residual *E. coli* DNA was less than 1% of the total DNA and was shown not to interfere with CMV PCR assays. This was demonstrated experimentally with 19 primer sets and was also demonstrated *in silico* with comparison of 72 CMV published primer sets with the *E. coli* DNA sequence, using BLAST.

DNA Sequencing of SRM 2366

Sequencing of the CMV SRM 2366 DNA is important, because the SRM will be used as a reference material in the assignment of values to calibrators of individual CMV qPCR assays. The target region for a given assay may or may not be

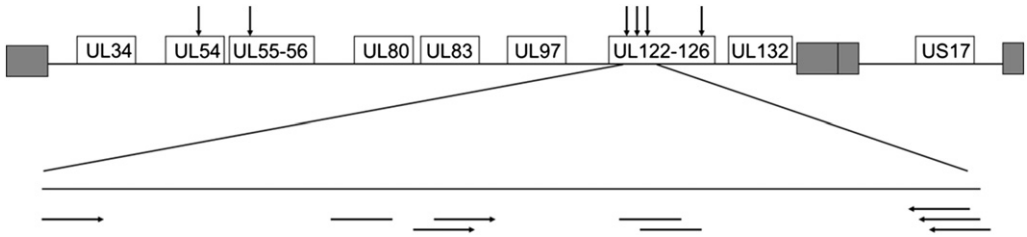

Figure 1 Schematic of the CMV genome. Regions sequenced are indicated by boxes. The dPCR assay locations for CP1, gBA, MIE 1F/1B, MIE B short, IE, and MIE 10 are indicated in order, left to right, by **vertical arrows**. The expanded view shows three dPCR assays that overlap each other (MIE 1F/1B, MIE B short, and IE), with primer binding sites indicated by **horizontal arrows** and probes indicated by **horizontal lines**. Not to scale.

highly conserved between laboratory strains and clinical isolates. Users of SRM 2366 need to know that their assay will be a match for the CMV DNA. Although it is convenient to know that there is a GenBank sequence for the strain of virus used to make the SRM, this knowledge is not sufficient, and the sequence of the SRM must be verified.

The genome of CMV is approximately 230,000 bp. Because of the large size of the genome, it was determined that only those regions that are used as targets for qPCR assays would be sequenced. This constituted approximately 7% of the genome (14,555 bp). The regions sequenced were locations from published qPCR assays (*UL54, UL55 to 56, UL83, UL122 to 126, and US17*) or commercial assays (*UL34, UL80, UL132*), or from mutation sites resulting in drug resistance (*UL97*) (Figure 1 and Table 2).

To increase the confidence in the sequence of the nucleotide ranges (Table 2), the sequences were independently verified by two individuals (Ross Haynes and Michael Coble). The sequenced regions match the Towne strain (GenBank accession number AY315197.2) exactly; one possible exception involves a single base in UL54 at position 78651, which shows both C and T peaks in the chromatogram. In particular, all three reverse strands show both C and T peaks in the chromatograms, whereas all four forward strands are clean in this region and show only a C peak in the chromatogram matching the Genbank sequence. It was determined that this particular nucleotide did not warrant further investigation, because notation of this potential ambiguity would be sufficient.

Table 2 Sequenced Regions of the CMV BAC Towne$_{\Delta 147}$

Region	Nucleotide range	Bases sequenced
UL34	43202–44971	1770
UL54	77695–79992	2298
UL55 to UL56	80848–82731	1884
UL80	114401–116793	2393
UL83	118890–119937	1048
UL97	140784–142090	1307
UL122 to UL126	170525–173182	2658
UL132	176380–177192	813
US17	198929–199312	384

Nucleotide ranges are based on GenBank accession number AY315197.2, which is the Towne strain. (Another CMV Towne strain submission, FJ616285.1, has slightly different numbering, and there is also a Towne-BAC sequence with the accession number AC146851.1.)

It should also be noted that the first 275 bases (nucleotide range, 176,380–176,654) of UL132 were sequenced entirely from reverse reads. There is a poly-A sequence immediately upstream of the region of this region of interest that causes a phase shift in both forward and reverse sequence reads and must be trimmed on the 3′ end of the poly-A stretch. One attempt to sequence through the poly-A sequence was made using another high-fidelity DNA polymerase (TaKaRa LA-Taq; Takara Bio, Otsu, Japan; Clonetech Laboratories, Mountain View, CA), but the phase shift beyond the poly-A stretch remained. To further verify the sequence of this portion of the region of interest, six different reverse primers were used to create 22 reverse reads in this portion of the region of interest with zero discrepant calls by the software or by either sequence reviewer.

Quantification of SRM 2366 Components

Quantitation of SRM 2366 in genome copies per volume (copies/μL) was accomplished separately for each of the three components, using dPCR. Tubes were sampled from three lots of each component and were assayed on multiple digital 12.765 arrays. This resulted in 99 data points for each component (75,735 total dPCR assays). Result of the analysis for components A, B, and C are shown, with the measurement uncertainty, in Table 3.

It was important to ascertain whether the assay target or amplicon size made a difference in the quantification. Assays were performed using six different primer and probe sets (Table 1). The distribution of the targets on the genome is illustrated in Figure 1. Three of the six assays targeting the major immediate early (MIE) gene region *UL122* overlapped with each other (Figure 1), and there was a difference in

Table 3 Values for the Three Components of SRM 2366 As Determined by Digital PCR

Component	Genome copies (no./μL)	Standard uncertainty (no./μL)	Relative uncertainty (%)	Expanded uncertainty (95% confidence interval)
A	420	56	13.3	301–523
B	1702	130	7.6	1446–1959
C	19,641	365	1.8	18924–20359

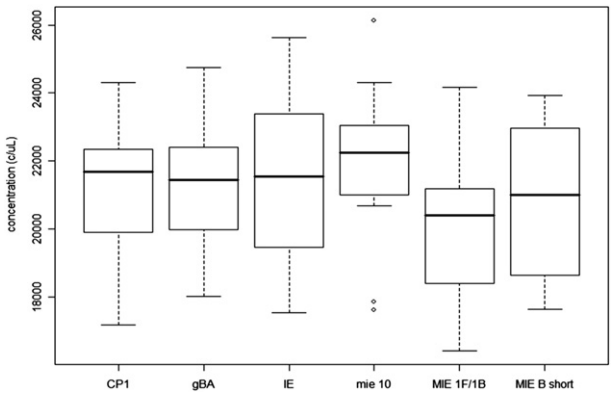

Figure 2 Comparison of the component C quantification results using six separate assays. Assays were CP1, gBA, IE, MIE 10, MIE 1F/1B, and MIE B short. Assays MIE 1F/1B (427 bp), MIE B short (138 bp), and IE (127 bp) target the same region but differ in size of the amplicon (Table 1 and Figure 1). All three overlap at one end of the gene target. All six assays were run on the same array, with each assay tested in two panels. Each box-and-whisker plot was constructed from 12 data points derived from six arrays.

amplicon size between MIE 1F/1B (427 bp) and the other two assays: MIE B short with 138 bp and IE with 127 bp. Assay comparison data are presented in Figure 2. The lowest mean value was seen with the assay that generated the largest amplicon (MIE 1F/1B, 427 bp), whereas the largest mean result was provided by the assay that generates a smaller amplicon (MIE 10, 86 bp). The other four assays had similar means, and ranged in amplicon size from 72 bp to 254 bp. A Gaussian mixed-effects linear model[31] was fitted to the data, with the assay as the fixed effect and the digital arrays as the random effect. Six different assays (CP1, gBA, MIE 1F/1B, MIE B short, IE, and MIE 10) and six different digital arrays were used, which gave 12 data points per assay type. There were no significant differences between the various assays. The P value of the F-test was 0.16. Thus, there was no evidence for a dependence on gene target or for amplicon size bias.

Stability of SRM 2366

Short-term stability testing is a stress test to measure how the material will behave during shipment. Because materials are more likely to experience increased temperatures during shipment than under conditions of normal laboratory storage and use, aliquots of each component were challenged at different temperatures (22°C, 37°C, and 65°C, with controls stored at 4°C) for different lengths of time (1, 2, and 4 weeks). All qPCR assays were performed synchronously at the end of the experiment. The analysis fits a regression over time and then tests whether the slope is 0. For the two lower temperatures, the test accepts the null hypothesis that the slope is zero; for the highest temperature, the test rejects the null hypothesis (Table 4). The results showed that the material is stable after 4 weeks at 22°C and 37°C, but not at 65°C. Long-term stability studies will be conducted on SRM 2366 during the 5-year life time of the SRM.

Table 4 Short-Term Stability

Temperature	Slope	95% Uncertainty interval for the slope
22°C	0.1	0.04 to 0.15
37°C	0.05	−0.07 to 0.17
65°C	0.43	0.24 to 0.62

Homogeneity of SRM 2366

Homogeneity testing is used to determine whether there are differences within each lot of the components. For example, measurement bias could be due to heterogeneity of the bulk materials or to some problem in the packaging process. Three random tubes chosen from each lot were assayed in triplicate by qPCR. No systematic trend between lots was observed (Figure 3). For all three components, therefore, all lots were recommended for release. The random-effects model,[31] used to determine the values and uncertainties of the SRM components, accounts for the variability between lots.

Interlaboratory Study

A total of 178 data sets for SRM 2366 were submitted by participants in the QCMD CMV EQA for 2010. Laboratory-developed assays were used to generate 78 data sets, and commercial assays were used to generate 100 data sets. The submitted values (in copies/mL) for component B of SRM 2366 were provided to NIST by QCMD. The consensus results were expressed in $\log_{10}$ copies per milliliter virus concentration with SD (Table 5). A combined plot of all of the data sets is presented in Figure 4. The kernel density estimate smoothes the data without losing the basic structure

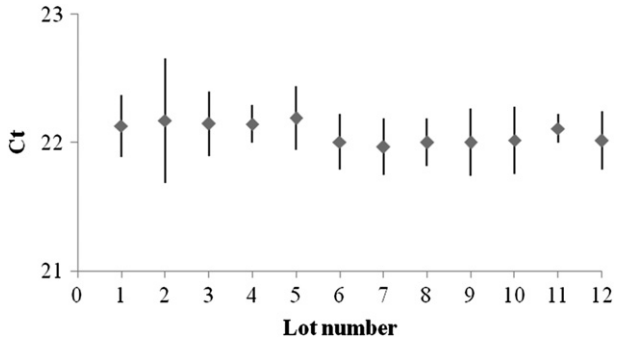

Figure 3 Homogeneity results for 12 lots of component C. Points are the mean C_T value; error bars encompass 95% confidence intervals. Each lot is defined as 100 consecutive tubes in the packaging process; therefore, any change in the material during filling should show as a trend on the graph. No systematic trend between lots was observed. The other two SRM 2366 components (A and B) were similarly tested, with similar results (data not shown).

Table 5 Quantitative Analysis by Type of Technology

QCMD CMV EQA participants and assays	Data sets (no.)	Mean	SD	Median	MADe
Total data sets	178	5.845	0.674	5.900	0.486
Conventional commercial	5	5.670	0.672	5.854	0.872
Real-time laboratory developed, total	78	5.909	0.756	6.002	0.650
Real-time commercial, total	95	5.795	0.600	5.826	0.451
Commercial kit A (1)	6	5.859	0.386	5.864	0.150
Commercial kit A (2)	15	6.224	0.345	6.205	0.332
Commercial kit B	21	5.632	0.880	5.733	0.794
Commercial kit C	28	5.767	0.321	5.821	0.326
Commercial kit D	12	5.789	0.233	5.776	0.298

Data are expressed as $\log_{10}$ copies per milliliter. The MADe statistic is an estimation of the SD of the median absolute deviation (MAD).

Interlaboratory study: Component B was included in the 2010 EQA for CMV by QCMD. Statistics were calculated only for groups that had ≥5 data sets, so the numbers for commercial kits A–D do not add up to the total number of commercial data sets.

of the data. In this case, the kernel density estimate is nearly identical to the Gaussian model (Figure 4). We also plotted the full width at half maximum (FWHM), as an estimation of the SD (Figure 4).

There were more outliers on the low end of values (Figure 4), and so more robust statistics; the median and MADe were also calculated for subgroup data sets (Table 5). The MADe statistic is an estimation of the SD of the median absolute deviation (MAD) and is suitable for use with near-normal data. With two exceptions, the median value for each grouping was slightly larger than the mean (Table 5).

The data were broken down by assay type, such as real-time commercial assay (Table 5). Five of the commercial kits had five or more data sets; the remaining seven had fewer than five data sets. Statistics were calculated for the groups that had five or more data sets. Observation of the medians (and means) indicated that the values were similar among the commercial products. The median for all of the laboratory-developed assays (6.002 $\log_{10}$ copies/mL) was similar to that for the commercial kits (5.826 $\log_{10}$ copies/mL).

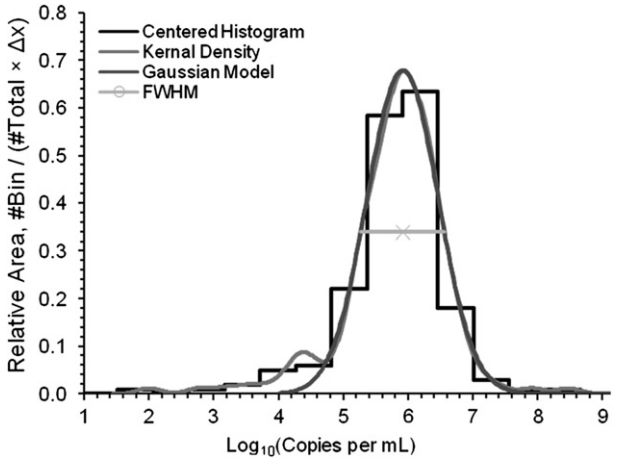

Figure 4 Histogram, kernel density, and Gaussian model for the SRM 2366 results from the QCMD 2010 CMV EQA. Note that the kernel density estimate is nearly identical to the Gaussian model. Full width at half maximum (FWHM) is an estimate of SD.

The MADe including all of the data sets was 0.48 $\log_{10}$ copies/mL, which is similar to, but somewhat higher than, the SD of all of the QCMD viral test samples (0.44 $\log_{10}$ copies/mL). In all groupings of the data, the consensus values for component B were lower than the concentration measured by dPCR (6.23 $\log_{10}$ copies/mL). The dPCR value (6.23 $\log_{10}$ copies/mL) certainly is well within the normal distribution (Figure 4). Whether the difference is statistically significant cannot be determined, because of the broad distribution of the data. The results of the study suggest that SRM 2366 is compatible with most assays currently in use.

Discussion

SRM 2366 became available in September 2011, and a World Health Organization International Standard for CMV became available from the National Institute for Biological Standards and Control (NIBSC, Potters Bar, UK; *http://www.nibsc.ac.uk*) in December 2010. Although the intent of usage for both standards is the same, the two are different materials, quantified differently, and the units are different (international units/volume versus copies/volume). SRM 2366 is a certified reference material (CRM); the World Health Organization standard is an international consensus calibrator. The approaches taken by the two institutions for the development of their respective standards have been recently reviewed in detail.[34,35] Studies are currently under way to determine the quantitative relationship of these two standards. There is room for both of these standards, and the molecular diagnostic community will need to decide how they can be best used to advance standardization of clinical diagnostic measurements.

Calibrants may consist of genomic DNA, plasmid DNA, or intact virus. Pure DNA calibrants can be directly compared with SRM 2366. The components of SRM 2366 have high concentrations of CMV DNA and can be diluted appropriately to make a standard curve for use in determining the concentration of the assay calibrants. Traceability

to SRM 2366 of calibrants that are part of commercial kits or stand-alone commercial calibrants should be established by the manufacturer. Measurement traceability for calibrants developed in laboratories that have their own laboratory-developed assays can be established by a similar procedure.

As a certified reference material, SRM 2366 can have a number of uses that could increase the quality of measurements, such as calibration, estimating method uncertainty, method validation/verification, quality control, or proficiency testing. However, the prime reason for production of SRM 2366 is its use as a primary reference material to establish metrological traceability for the calibrants that are used in the many CMV qPCR assays. Metrological traceability is defined in the International Vocabulary of Metrology (VIM)[36] as a "property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty." Documentary standards can provide some guidance on how to conduct the appropriate traceability measurements. Of particular interest are the ISO 17511:2003 and ISO 15194:2009 standards, with their supporting documentation,[37,38] as well as CLSI X5-R.[39] Although there is not a precise protocol outlined in the documentary standards that fits the case, there are examples that can be adapted, such as in Appendix C of the CLSI X5-R report.[39] A recent review discusses documentary standards.[34]

There are two compelling reasons for standardizing CMV PCR viral load assays. First, the community uses a variety of assays (with both laboratory-developed and commercial reagents) and a variety of calibrants (again, both laboratory-developed and commercial). The QCMD EQA program for CMV in 2011 reported that, of 215 data sets, 61% were generated using commercial reagents and kits and 39% were generated using laboratory-developed tests. How many unique assays were represented by the data sets is not known, nor the different combinations of extraction methods that need to be considered. In the 2011 EQA, 19 different commercial assay types were used, with 1 to 22 data sets attributable to the various commercial reagents and kits. The second reason is related to the first, in that an individual patient with CMV disease or CMV DNAemia may be monitored over a period of weeks, months, or more than 1 year (for late CMV disease). It has been suggested that the pattern over time of increase or decrease in viral load is important, not only the absolute values.[15] Although in a perfect world all monitoring over time would be done with the same assay in the same laboratory, in practice individual patients may be tested through multiple laboratories. Standardization of both assays and calibrants in principle would help reduce the interlaboratory variability of CMV PCR viral load assays. Standardization of calibrants to an SI-traceable certified reference material such as SRM 2366 can help ensure that measurement results from these assays can be confidently compared over time and place.

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