Methods 201 (2022) 65–73

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

The performance of human cytomegalovirus digital PCR reference measurement procedure in seven external quality assessment schemes over four years

Mojca Milavec[a,*], Jernej Pavšič[a], Alexandra Bogožalec Košir[a], Gerwyn M. Jones[b], Denise M. O'Sullivan[b], Alison S. Devonshire[b], Fran Van Heuverswyn[d,1], Maria Karczmarczyk[d,2], Jannika Neeb[e,3], Annabell Plauth[e], Philippe Corbisier[d], Heinz Schimmel[d], Andreas Kummrow[e], Jörg Neukammer[e,4], Carole A. Foy[b], Martin Kammel[f,g], Hans-Peter Grunert[h], Heinz Zeichhardt[f,g,h], Jim F. Huggett[b,c]

[a] Department of Biotechnology and Systems Biology, National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia
[b] National Measurement Laboratory (NML), LGC, Queens Road, Teddington, Middlesex TW11 0LY, United Kingdom
[c] School of Biosciences & Medicine, Faculty of Health & Medical Science, University of Surrey, Guildford, United Kingdom
[d] European Commission, Joint Research Centre (JRC), Geel, Belgium
[e] Physikalisch Technische Bundesanstalt, Abbestr. 2-12, D-10587 Berlin, Germany
[f] INSTAND, Gesellschaft zur Foerderung der Qualitaetssicherung in medizinischen Laboratorien e.V., Ubierstr.20, D-40223 Düsseldorf, Germany
[g] IQVD GmbH, Institut fuer Qualitaetssicherung in der Virusdiagnostik, Potsdamer Chaussee 80, D-14129 Berlin, Germany
[h] GBD Gesellschaft fuer Biotechnologische Diagnostik mbH, Berlin, Potsdamer Chaussee 80, D-14129 Berlin, Germany

ARTICLE INFO

Keywords:
Digital PCR
Human cytomegalovirus
External quality assessment
Reference measurement procedure

ABSTRACT

A candidate digital PCR (dPCR)-based reference measurement procedure for quantification of human cytomegalovirus (hCMV) was evaluated in 10 viral load comparison schemes (seven external quality assessment (EQA) and three additional training schemes) organized by INSTAND e.V. over four years (between September 2014 and March 2018). Four metrology institutes participated in these schemes using the same extraction method and dPCR measurement procedure for the hCMV specific target sequence of *UL54* gene. The calibration independent reference measurement procedure results from the metrology institutes were compared to the results of the clinical diagnostic laboratories applying hCMV qPCR measurement procedures calibrated to reference materials. While the criteria for the acceptable deviation from the target value interval for INSTAND's EQA schemes is from $-0.8 \log_{10}$ to $+0.8 \log_{10}$, the majority of dPCR results were between $-0.2 \log_{10}$ to $+0.2 \log_{10}$. Only 4 out of 45 results exceeded this interval with the maximum deviation of $-0.542 \log_{10}$. In the training schemes containing samples with lower hCMV concentrations, more than half of the results deviated less than $\pm 0.2 \log_{10}$ from the target value, while more than 95% deviated less than $\pm 0.4 \log_{10}$ from the target value. Evaluation of intra- and inter-laboratory variation of dPCR results confirmed high reproducibility and trueness of the method. This work demonstrates that dPCR has the potential to act as a calibration independent reference measurement procedure for the value assignment of hCMV calibration and reference materials to support qPCR calibration as well as ultimately for routine hCMV load testing.

* Corresponding author.
E-mail address: mojca.milavec@nib.si (M. Milavec).

[1] Flanders Vaccine, vzw, Agoralaan building A bis, B-3590 Diepenbeek, Belgium.
[2] Eurofins Food Testing UK Limited, i54 Business Park, Valiant Way, WV9 5GB, Wolverhampton, United Kingdom.
[3] Technische Universität Berlin, Faculty III Process Sciences, Institute of Biotechnology, Straße des 17. Juni 135, 10623 Berlin, Germany.
[4] Charité - Universitätsmedizin Berlin, Institute of Transfusion Medicine, 10117 Berlin, Germany.

https://doi.org/10.1016/j.ymeth.2021.03.016
Received 28 January 2021; Received in revised form 18 March 2021; Accepted 22 March 2021
Available online 1 April 2021
1046-2023/© 2021 The Author(s). Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Molecular methods for detection and quantification of nucleic acids are increasingly being used as diagnostic tools by laboratories in a variety of medical fields. As well as assessment of human sequences, to aid human genetics and increasingly cancer management, infectious diseases diagnostics apply molecular methods for detection of the causative agents, potential antidrug resistance, and estimation of pathogen loads before and during the treatments [1–3]. One of the most commonly used molecular diagnostic methods is the quantitative polymerase chain reaction (qPCR) applied as commercial kits and in-house developed methods. While there are many examples of successfully applied molecular tests, as their use becomes more demanding, such as measuring quantity of multiple pathogens, the challenges associated with reproducible measurements increase. Many technical factors, including specimen type, sampling method, nucleic acid extraction, calibration and qPCR reaction components, can lead to discrepancies between results within or among clinical laboratories that use qPCR-based tests to quantify pathogen load. Consequently, laboratory may inaccurately assess the pathogen burden and lead to incorrect clinical decision.

To account for such technical discrepancies diagnostic laboratories can assess their methods by participating in organized inter-laboratory comparisons, such as External Quality Assessment (EQA) schemes [4,5]. A prerequisite for reliable comparison among laboratories are stable, homogenous, and ideally commutable, materials of known composition. Depending on the analyte, EQA materials may be value assigned by reference methods, by comparison to other reference materials, or through the calculation of consensus of participants' results [6,7]. One of the challenges in EQA schemes for molecular analysis of infectious agents is determining the concentration values of the materials. Due to the absence of reference methods and limited availability of reference materials in the field of nucleic acid based analysis of infectious agents, a ''target value'' is determined using consensus of participants' results [6]. Depending on the infectious agent, variability of participants' results can be very high.

In case of the INSTAND EQA for virus genome detection of human Cytomegalovirus (hCMV), target value is calculated from participating laboratories results which can vary for more than 100-fold [8–15]. This variability is higher compared to other frequently quantified viruses like HIV, and hepatitis B and C and differences in results from different laboratories using qPCR persist in spite of availability of two global standards. The 1st WHO International Standard for Human Cytomegalovirus for Nucleic Acid Amplification Techniques (09/162) has been available since 2010, and provides an assigned concentration of $\sim 5 \times 10^6$ International Units (IU) per mL based on the results of a worldwide collaborative study [16]. NIST Standard Reference Material 2366 Cytomegalovirus (CMV) for DNA Measurements has been available since 2011 and consists of three components with different concentrations of genome copies per μL (cp/μL) [17]. These standards can serve for value assignment of secondary reference materials for the development of traceable quantitative calibrators, and secondary standards for method validation or daily controls [2].

Since the introduction of the 1st WHO International Standard for Human Cytomegalovirus in 2010 the number of commercial kits enabling reporting in IU/ml has slowly increased. However, the introduction of the standard has not led to a reduction of variation in the hCMV quantification in the INSTAND EQA schemes expressed in an overall success rates (fraction of laboratories with correct results for all samples) which remain between 62.8% [18] and 100% [9] for a ± 0.8 $\log_{10}$ target range interval for results in IU/ml in the EQA schemes between 2014 and 2017 (with an average success rate of 86.6%). This is in contrast to other clinical viral targets like HBV, where with only a ± 0.6 $\log_{10}$ target range interval the overall success rates in IU/ml are between 92.0% [19] and 98.5% [20]. Moreover, laboratories are reporting their results in different units, IU or copies per unit volume.

Recognizing the need to support hCMV quantification and lack of reference methods, we developed and evaluated qPCR and digital PCR (dPCR) based methods for quantification of *UL54* gene, which codes for hCMV DNA polymerase [21]. Due to its higher tolerance to inhibitors, high reproducibility and simpler calibration, dPCR was selected for further evaluation, developed and validated as a reference measurement procedure [22]. While standards for hCMV are available for almost a decade, this reference measurement procedure to value assign the concentration of hCMV genomic DNA in solution was internationally accepted only very recently by Joint Committee for Traceability in Laboratory Medicine (JCTLM) and has not been integrated into medical guidelines yet. The method is described here in its application within seven EQA schemes ''Virus Genome Detection – Cytomegalovirus'' (INSTAND code 365) and in three additional schemes ''Virus Genome Detection – Cytomegalovirus – Additional Training Program'' (INSTAND code 368) between September 2014 and March 2018. Three national metrology institutes or designated institutes, as well as the Joint Research Centre (JRC-GEEL) (from here on, "metrology laboratories") have participated in these schemes providing data for the evaluation of the reproducibility and trueness of the developed method.

2. Materials and methods

The "Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020" (dMIQE2020) checklist [23] is given in (Supplementary Table S1).

2.1. INSTAND's EQAs

INSTAND e.V. is a reference institution of the German Medical Association and conducts EQA schemes for various areas of medical laboratory diagnostics. INSTAND's EQAs are organized according to the basic principles of internal and external quality assurance specified in the Guidelines of the German Medical Association on quality assurance in medical laboratory testing (Rili-BÄK) [6,24]. The detection of concentration of hCMV nucleic acids is one of the virus diagnostics specified for external quality assessment and subject to mandatory participation in the corresponding EQA schemes in Germany. It has been provided since 2003. The EQA scheme "Virus Genome Detection – Cytomegalovirus" (INSTAND code 365) is organized four times per year, with samples containing virus concentrations within the requirements stated in the Rili-BÄK and results within an interval of $-0.8 \log_{10}$ to $+0.8 \log_{10}$ in respect to the target value are considered as being consistent.

In addition to the main EQA scheme a training program for virus genome detection "Cytomegalovirus – Additional Training Program" (INSTAND code 368) is available once per year. It contains low-concentration samples to verify test linearity for concentration values approaching the limit of quantification. The low-concentration samples are directly linked to the main EQA scheme (INSTAND code 365) within the corresponding EQA scheme term by application of an overlapping sample. The target value of a given EQA scheme sample is derived from the consensus value (based on the robust average according to algorithm A/DIN ISO 13528/Annex C) [25] from all quantitative and qualitative results reported by the participants. All reports can be found at: https://www.instand-ev.de/no_cache/en/eqas-online/service-for-eqa-tests/.

2.2. EQA samples and sample pre-treatment

hCMV-positive samples used in EQAs were plasma pools of healthy blood donors spiked with a lysate of hCMV infected cells (isolate of a patient) and hCMV-negative samples consisted of negative plasma pool of healthy blood donors [9–15,26–35]. Each laboratory received 2 or 3 units (tubes) of each sample (lyophilized plasma). After receipt, the samples were stored in refrigerators (+2 °C to +8 °C) until analysis. The lyophilized samples were reconstituted in 1.1 mL water (sterile, pyrogen-free, PCR-grade) directly before analysis according to

Instructions for Test Performance [26–28,30,32,33,35]. All evaluated hCMV-positive samples are listed in the supplement (Supplementary Tables S2 and S3).

2.3. DNA extraction and dPCR in metrology laboratories

One or two 200 μL aliquots were prepared from each sample unit and extracted, using High Pure Viral Nucleic Acid kits (Roche), according to the manufacturer's instructions. The extracted gDNA was either directly analyzed or stored at – 20 °C for further use. Negative extraction controls were included in each experiment.

hCMV DNA was quantified using an assay targeting a specific sequence of the *UL54* gene [21,36]. 20 μL reactions were used, composed of 10 μL 2× ddPCR Supermix for Probes (Bio-Rad Laboratories, USA); primers and probe mix (600 nM primers/200 nM probe); 7 μL or 8 μL sample and nuclease-free water. Negative template controls were included in each experiment. Positive control templates (e.g. hCMV strain AD-169 genomic DNA at approx. 5000 cp/μL (ATCC VR-538) or positive samples from previous studies) were used in some experiments. The reactions were performed in duplicates or triplicates in 96-well plates using universal PCR conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C and 10 min at 98 °C at the end of the cycling. Ramp rate was set to 2 °C/s. One laboratory did not use the first step, 2 min at 50 °C, of PCR cycling and repeated 40 cycles only. The reactions were performed on the GeneAmp® PCR System 9700 (ABI), T100 (Bio-Rad) and C1000 (Bio-Rad). After the PCR step, the 96-well plates were loaded onto QX100/200 droplet readers. The analysis of the droplet fluorescence was done with QuantaSoft Software versions 1.3.2.0, 1.6.6.320 and 1.7.4.0917 and QuantaSoft Pro 1.0.596 (all Bio-Rad). The fluorescence was monitored over the FAM spectral region. All of the thresholds were set up manually to allow the distinction between positive and negative droplets. Only the reactions with more than 10,000 accepted droplets were used for analysis and reactions with less than 3 positive droplets were considered negative. Limit of detection of the method has been set to 5.7 copies per total reaction volume [21].

2.4. DNA extraction and qPCR in other participating laboratories

Other laboratories participating in the EQA scheme for hCMV genome detection utilize extraction methods routinely used in detection of hCMV from various providers (Abbott, BioMerieux, ELITech, Chemagen, GeneProof, Macherey-Nagel, Promega, Qiagen, Roche, Sacace Biotechnologies, Siemens), and different kits or in-house developed nucleic acid amplification methods as well as different qPCR platforms. In some participating laboratories, automated methods have been used combining DNA extraction and qPCR (for example, COBAS AmpliPrep/COBAS TaqMan CMV test (Roche)). Manufacturers, tests and equipment are provided in the Supplementary material (Table S4).

2.5. Data analysis

Metrology laboratories reported all results in cp/mL of reconstituted samples including expanded measurement uncertainty, corresponding to a 95% confidence interval for the submitted value. Standard measurement uncertainties, equivalent to within-laboratory precision for a stipulated level of replicate unit and technical (dPCR) measurement, were approximated by dividing the expanded uncertainty by the coverage factor ($k = 2$). Relative expanded uncertainties (%) correspond to the uncertainty in cp/mL divided by the submitted value.

Other laboratories participating in 'Virus Genome Detection – Cytomegalovirus'' (INSTAND code 365) and ''Virus Genome Detection – Cytomegalovirus – Additional Training Program'' (INSTAND code 368) EQA schemes reported their results in IU/mL or in cp/mL or in both.

In this paper, all results from EQA schemes reported in cp/mL were tested for normality of the distribution using Shapiro Wilk test (Descriptive statistics and Normality, Real Statistics, Excel 2016) and $\log_{10}$ transformed. Transformed data was used to evaluate the deviations from the target values.

3. Results

Four metrology laboratories (up to three per scheme or program) participated in seven INSTAND EQA schemes for Virus Genome Detection – Cytomegalovirus (code 365) and three training programs (code 368) between 2014 and 2018. These laboratories were using the same validated extraction method and the same dPCR measurement method for hCMV specific target sequence of *UL54* gene. They reported the results in copies per mL (cp/mL) and their results were compared to the results of other participants reporting in cp/mL measured with qPCR using various tests (Supplementary Table S4).

3.1. Virus Genome Detection – Cytomegalovirus schemes (INSTAND code 365)

In the INSTAND EQA scheme for virus genome detection of cytomegalovirus (INSTAND code 365) four samples with different sample properties were provided. Target hCMV concentrations – calculated as a consensus from all reported quantitative results in cp/mL – ranged from 2037 to 12,194,816 cp/mL. In seven Virus Genome Detection – Cytomegalovirus schemes between 46 and 64 participants, including the metrology laboratories, reported results in cp/mL. All results reported by metrology laboratories based on dPCR measurements were within the acceptable deviation, namely within interval from $-0.8 \log_{10}$ to $+0.8 \log_{10}$ (Fig. 1). Metrology laboratories determined all hCMV negative samples (pool of plasmas from healthy blood donors) as negative.

In five out of seven main EQA schemes the maximum deviations of metrology laboratories dPCR results were below $\pm 0.25 \log_{10}$. Higher deviations of dPCR results were observed in June 2017 EQA scheme [12] and November/December 2017 EQA scheme [14] (Fig. 1) and may be connected to sample properties. In June 2017 EQA scheme samples *365113* and *365116* represented different dilution steps at the extremes of the tested concentration range (10^7 and 10^3 cp/mL respectively), while sample *365115* was from a different source. Maximum deviations for samples *365113* and *365116* were $-0.335 \log_{10}$ and $-0.542 \log_{10}$, respectively, while for the sample *365115* (target value 3789.2 cp/mL) the deviation was very low, less than $-0.08 \log_{10}$. In the November/December 2017 EQA results with dPCR showed very high deviation with the lowest hCMV level, $0.353 \log_{10}$ for sample *365121* (target value 4554 cp/mL). In addition, measurement uncertainty corresponding to a 95% confidence interval was high for all samples in this EQA scheme (67–97% relative expanded uncertainty) (Supplementary Table S2).

Samples *365073* and *365076*, provided in November / December 2014 EQA scheme [10], were identical (Fig. 1). Comparison of results of identical samples showed that the majority of results were within the acceptance interval (Fig. 2).

However, in several laboratories discrepancies between the results from the two samples were observed, indicating suboptimal reproducibility. On the other hand, with dPCR measurements the results of these two samples were in better agreement than in the majority of other laboratories.

3.2. Virus Genome Detection Cytomegalovirus – Additional Training Program (INSTAND code 368)

The participation in the INSTAND EQA scheme Virus Genome Detection – Cytomegalovirus – Additional Training Program (INSTAND code 368) is not obligatory for medical laboratories in Germany and thus the number of participants is lower compared to the number of participants in Virus Genome Detection – Cytomegalovirus (INSTAND code 365). In the three training programs described below there were up to 14 participants, including metrology laboratories (Supplementary

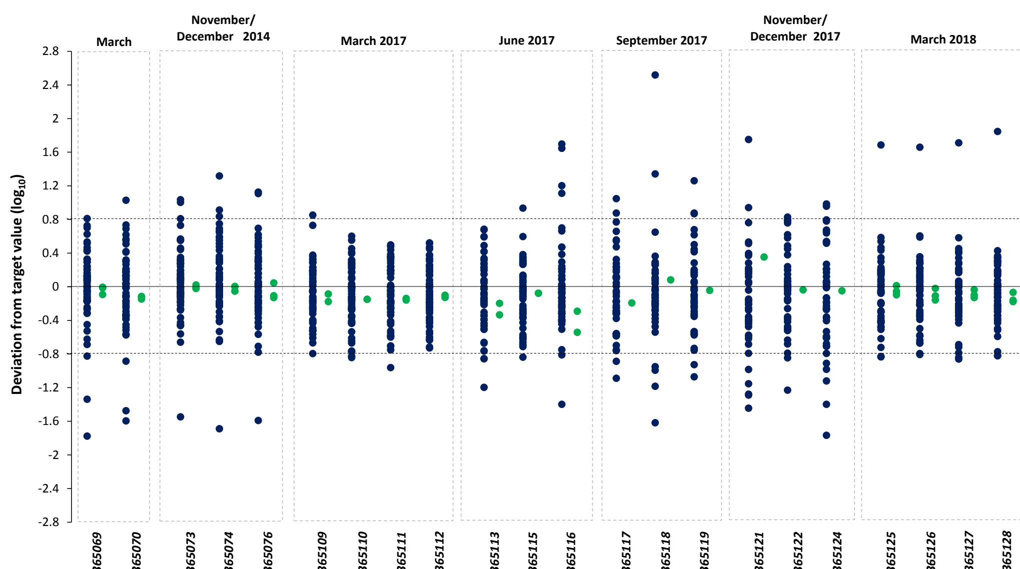

Fig. 1. Results for hCMV positive samples (sample numbers *365069–365128*) of seven INSTAND EQA schemes performed between September 2014 and March 2018 presented as deviations of laboratory results ($\log_{10}$) from the target (consensus) value calculated as the mean of all results in particular EQA scheme Virus Genome Detection – Cytomegalovirus (INSTAND code 365); blue dots – qPCR, green dots – dPCR; ±0.8 $\log_{10}$ (dashed line) is the acceptable deviation of a quantitative result measured by an individual laboratory with respect to the target value. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

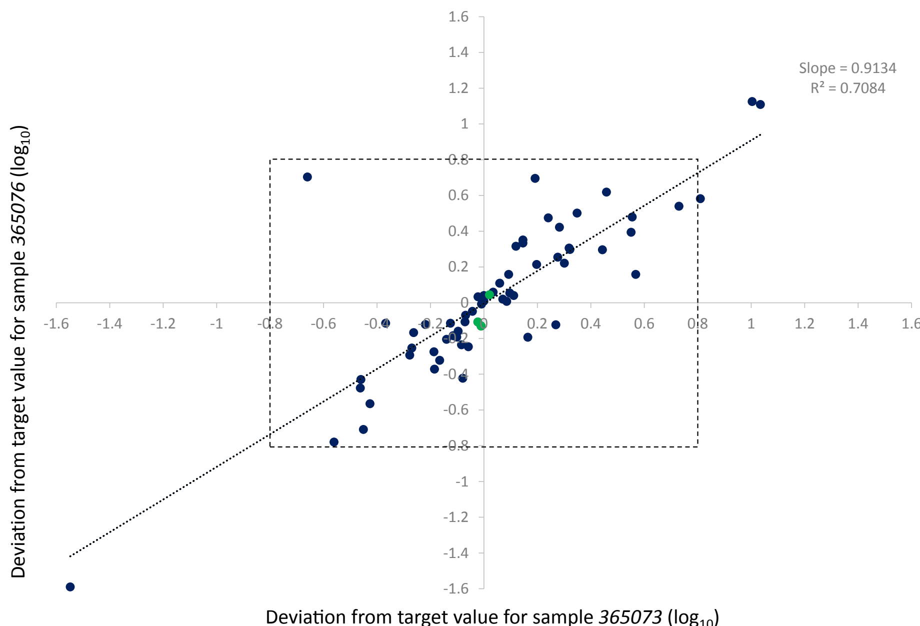

Fig. 2. Comparison of laboratory results ($\log_{10}$) of the two identical samples from EQA scheme Virus Genome Detection – Cytomegalovirus (365) November/December 2014; blue dots – qPCR, green dots – dPCR; ±0.8 $\log_{10}$ (dashed line) is the acceptable deviation of a quantitative result measured by an individual laboratory in respect to the target value. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table S3). In the Cytomegalovirus – Training Program (368) schemes four samples with different sample properties are provided. The concentration of hCMV in hCMV positive samples is lower than in the samples of the corresponding main EQA scheme (INSTAND code 365). The target concentrations of hCMV – calculated as a consensus from all reported quantitative results in cp/mL – of these training samples were between 42 and 8278 cp/mL. With the exception of one result, all results reported by metrology laboratories based on dPCR measurements were within the acceptable deviation, namely within interval from -0.8 $\log_{10}$ to $+0.8$ $\log_{10}$ (Fig. 3). Metrology laboratories determined all hCMV negative samples (pool of plasmas from healthy blood donors) as negative.

The deviations of dPCR results from the target value were higher in the training programs compared to the main EQA schemes. Only in September 2014 Cytomegalovirus – Training Program (368) [34] the deviation from the target value was below ±0.25 $\log_{10}$ for all results from metrology laboratories, with one exception for very low hCMV level sample *368007* (target value 42 cp/mL) (Fig. 3). In EQA scheme Virus Genome Detection – Cytomegalovirus – Additional Training Program in March 2017 [29] maximum deviation from the target value was -0.376 $\log_{10}$ for the sample *368022* with the lowest hCMV concentration of 532 cp/mL, while in March 2018 [31] maximum deviation from the target value was -0.286 $\log_{10}$ for the sample *368026* with the highest hCMV concentration of 6271 cp/mL.

Each training program was directly linked to the corresponding main EQA scheme through an overlapping (identical) sample (Fig. 4). In September 2014 the overlapping sample was marked *365071* in the main EQA scheme and *368005* in the training program [34]. Comparison of results for the overlapping samples measured in both studies showed a high coefficient of determination indicating good intermediate

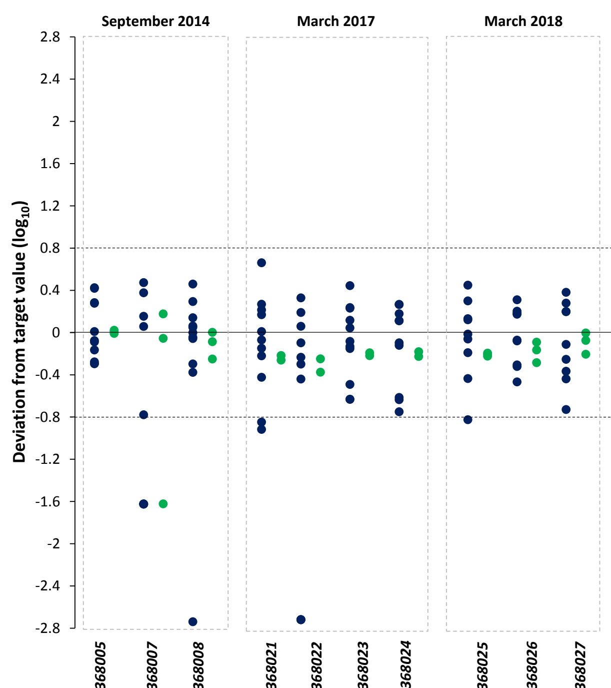

Fig. 3. Results for hCMV-positive samples (sample numbers *368005–368027*) of the three training schemes performed between September 2014 and March 2018 presented as deviations of laboratory results (log_{10}) from the target (consensus) value calculated as the mean of all results in particular EQA scheme Cytomegalovirus – Training Program (368); blue dots – qPCR, green dots – dPCR; ±0.8 log_{10} (dashed line) is the acceptable deviation of a quantitative result measured by an individual laboratory with respect to the target value. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

precision for all labs (Fig. 4A). While in the main EQA scheme the target value for the overlapping sample was 11,034 cp/mL (calculated from 64 results) in the training program the target value was 8278 cp/mL (calculated from 14 results). Results of measurements at metrology laboratories obtained with dPCR were similar in both studies. They were ranging from 8116 cp/mL to 8687 cp/mL in the main EQA scheme (Supplementary Table S2) and from 7860 cp/mL to 8459 cp/mL in the training program (Supplementary Table S3), leading to higher deviations of the results from the target value in the main EQA study (Fig. 4A). In March 2017 the overlapping sample was marked *365109* in the main EQA scheme and *368021* in the training program [29]. Target value of the overlapping sample was 8742 cp/mL (calculated from 57 results) in the main EQA scheme, while in the training scheme its target value was 12,196 cp/mL (calculated from 11 results). Results obtained with dPCR were reproducible, 7152 cp/mL in the main EQA scheme and 7373 cp/ml in the training program in one laboratory, and 5800 cp/ml and 6690 cp/ml, respectively, in the other laboratory, leading to higher deviations of the results from the target value in the main EQA study and a low coefficient of determination in the correlation plot (Fig. 4B).

In the last scheme, in March 2018, the overlapping sample was marked *365127* in the main EQA scheme and *368109* in the training program [31]. Target value of the overlapping sample was 2037 cp/mL (calculated from 53 results) in the main EQA scheme, while in the training scheme its target value was 2475 cp/mL (calculated from 11 results). Results obtained for the overlapping sample in three metrology laboratories using dPCR measurements were comparable and below both target values (Fig. 4C, Supplementary Tables S2 and S3).

3.3. dPCR Intra- and Inter-laboratory variation

Results obtained by the metrology laboratories using dPCR in the

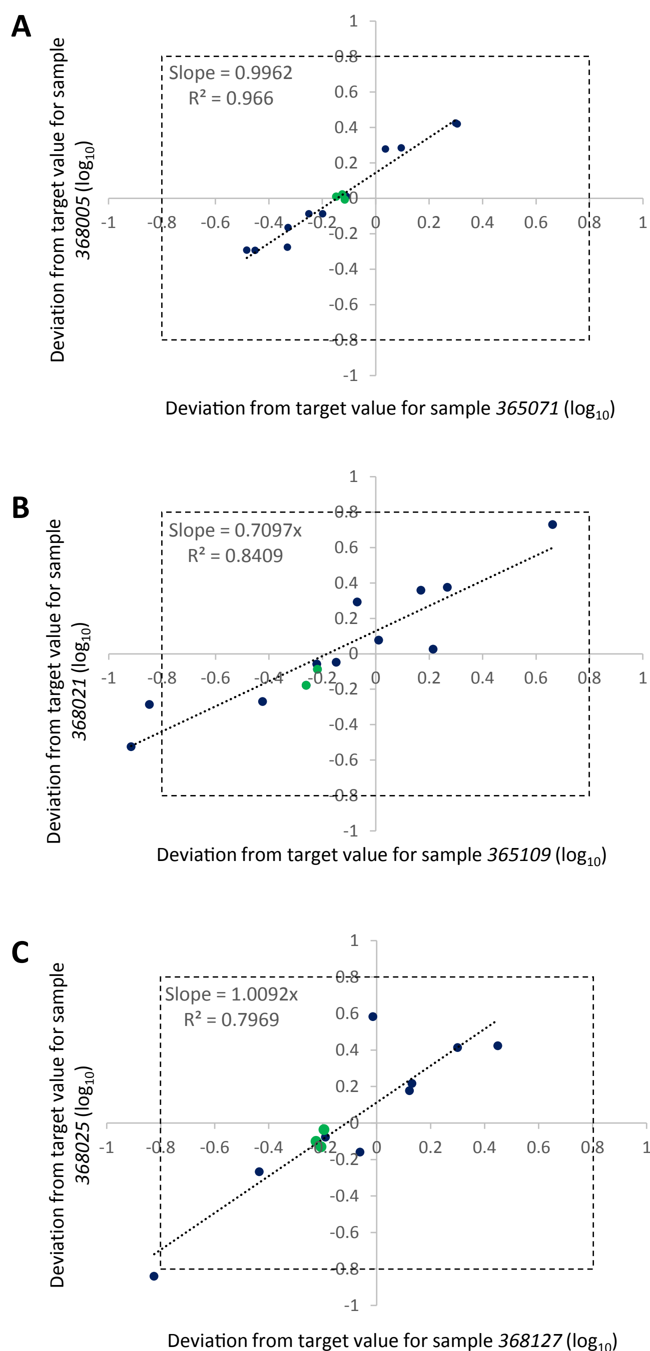

Fig. 4. Comparison of laboratory results (log_{10}) of the overlapping (identical) samples from EQA schemes Virus Genome Detection – Cytomegalovirus (365) September 2014 and Virus Genome Detection – Cytomegalovirus – Additional Training Program (368) September 2014 A; Virus Genome Detection – Cytomegalovirus (365) March 2017 and Virus Genome Detection – Cytomegalovirus – Additional Training Program (368) March 2017 B; Virus Genome Detection – Cytomegalovirus (365) March 2018 and Virus Genome Detection – Cytomegalovirus – Additional Training Program (368) March 2018 C; blue dots – qPCR, green dots – dPCR; ±0.8 log_{10} (dashed line) is the acceptable deviation of a quantitative result measured by an individual laboratory with respect to the target value. Target values for particular EQA scheme were calculated as a consensus from all reported quantitative results in cp/ml. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

main EQA schemes were further evaluated in terms of intra-and inter-laboratory variation. For three EQA schemes (September and November 2014 and March 2018) where a minimum of three metrology laboratories participated, intra-laboratory variation, expressed as relative standard uncertainty, was generally below 20% in concentration range between 10^3 cp/mL and 10^6 cp/mL and below 35% for concentrations between 10^2 cp/mL and 10^3 cp/mL (Fig. 5A). Inter-laboratory variation expressed as CV (n = 3) was ≤16% in the concentration range 10^4 cp/mL to 10^6 cp/mL and less than 25% in concentration range between 10^2 cp/mL and 10^3 cp/mL (Fig. 5B). Regression analysis of results from the two metrology laboratories (NML, NIB) that have participated in five common EQA schemes showed good agreement over a concentration range spanning five decades (Fig. 5C), confirming excellent reproducibility of the measurement procedure including extraction.

4. Discussion

Evaluation of dPCR measurement results from seven EQA schemes ''Virus Genome Detection – Cytomegalovirus'' (INSTAND code 365) and in three schemes ''Virus Genome Detection – Cytomegalovirus – Additional Training Program'' (INSTAND code 368) showed lower deviations from target values (defined as consensus values) compared to majority of qPCR results. While the acceptable deviation from the target value interval for INSTAND's EQAs is from -0.8 $\log_{10}$ to $+0.8$ $\log_{10}$, the majority of dPCR results was between -0.2 $\log_{10}$ to $+0.2$ $\log_{10}$ in ''Virus Genome Detection – Cytomegalovirus'' (INSTAND code 365). Only 4 out of 45 dPCR results exceeded this interval with the maximum deviation of -0.542 $\log_{10}$. In the EQA scheme ''Virus Genome Detection – Cytomegalovirus – Additional Training Program'' (INSTAND code 368) more than half results deviated less than ±0.2 $\log_{10}$ from the target value, while more than 95% deviated less than ±0.4 $\log_{10}$ from the target value.

Metrology laboratories were using the same extraction method and the same nucleic acid amplification method; however, they were not using any calibration in their measurements. The dPCR measurement used by metrology laboratories in this study has been previously compared to qPCR [21] and the whole procedure, including extraction, has been evaluated in a small inter-laboratory comparison [22]. It has also been tested for direct amplification of 1st WHO standard and EQA samples [37]. All previous comparisons and the present evaluation, led to a well evaluated method for quantification of purified hCMV genomic DNA in solution, which has been listed in the database of reference measurement methods of the Joint Committee for Traceability in Laboratory Medicine (JCTLM) in July 2020 (https://www.bipm.org/jctlm/). It is only the second reference measurement method for nucleic acids listed in the JCTLM database and the first on infectious agents.

The reproducibility of the dPCR measurements was confirmed in EQA studies with overlapping samples. The dPCR results obtained are close to the averaged results of qPCR with a minute tendency to determine lower concentrations, which may be the result of the calibrators used in qPCR. While measurement results of these EQA samples were reproducible, the deviations from target values of overlapping samples were different due to the different numbers of participants' results used for target value calculations in the main EQA schemes compared to the training programs leading to variable target values. The majority of measurements of samples from the main EQA schemes showed low variance with measurement uncertainties and 95% confidence intervals below 30%. Variance of measurement values was higher in training programs with lower hCMV concentrations. Higher variance of dPCR measurement results in some main EQA schemes (e.g. September 2017 and November/December 2017) could be connected to suboptimal reconstitution or homogeneity of samples. Namely, metrology laboratories were using one or two times 200 μL subsamples in extraction procedures and measured the quantity of hCMV DNA in duplicates or triplicates.

For standardization of nucleic acid amplification technique-based

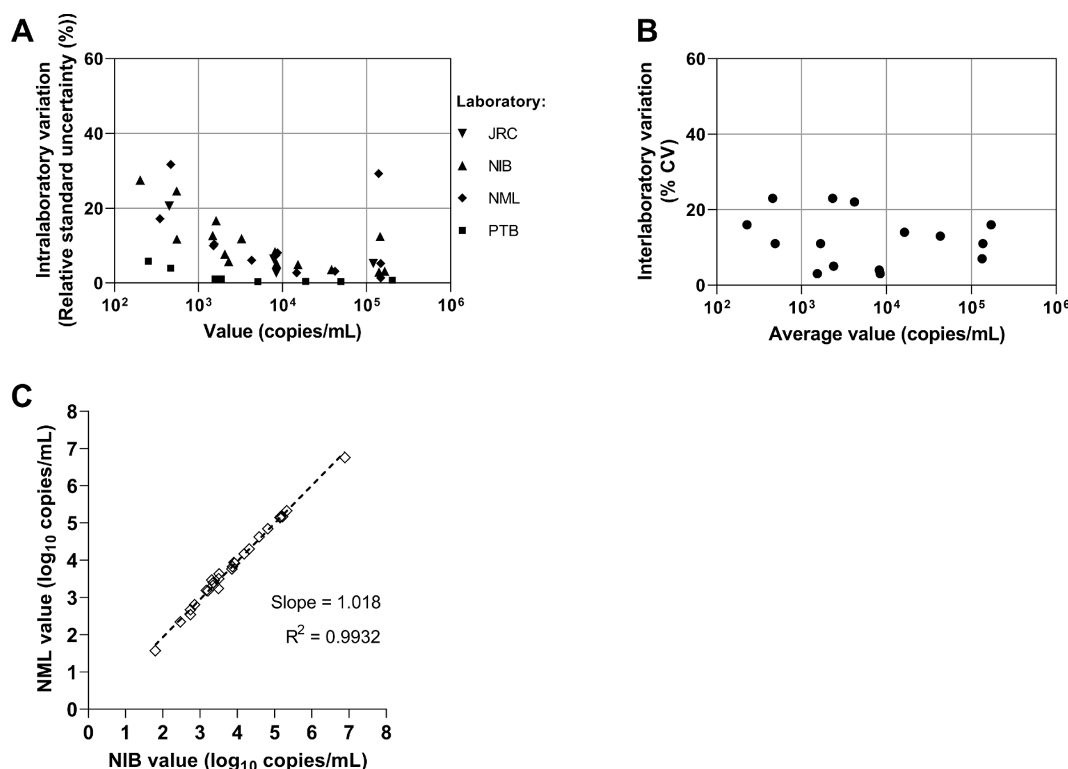

Fig. 5. dPCR Intra- and inter-laboratory variation. A-B: Intra- and inter-laboratory variation of metrology laboratories' dPCR results are plotted against the magnitude of the copy number concentration (copies/mL) individual or average result, shown for schemes where three metrology laboratories participated. C: Comparison of results for all schemes where both NIB and NML participated; linear regression analysis (dashed line) applies to $\log_{10}$-transformed copy number concentration (copies/mL) results.

assays for hCMV, 1st WHO International Standard for Human Cytomegalovirus for Nucleic Acid Amplification Techniques NIBSC, code: 09/162, was developed. This material has an assigned value of $\sim 5 \times 10^6$ International Units (IU) [16]. The value has been assigned based on the results of a worldwide collaborative study and is valid when the lyophilized material is reconstituted in 1 mL of nuclease-free water. In our previous studies this standard has been measured by dPCR measurement procedure used in this study providing concentration in copies per mL either after the extraction of standard spiked in human plasma (4.46×10^6 cp/mL) or in buffer (3.9×10^6 cp/mL) or by direct quantification (5.25×10^6 cp/mL) [37]. Due to the availability of this standard and in line with the Specified Section B3 (Table B 3-2a, column 4) of the Guideline of the German Medical Association for quality assurance of medical laboratory analyses [6] in INSTAND's EQA schemes primarily IU/mL are considered as the measurement unit for reporting quantitative results of genome detection of hCMV. However, the participating laboratories are reporting in line with specifications of amplification kits they are using. For example, participants using Abbott RealTi*m*e CMV assay (Abbott) are reporting in IU/mL as this kit is standardized to the 1st WHO International Standard for Human Cytomegalovirus, while participants using artus® CMV PCR Kit (Qiagen) are reporting in cp/mL as this kit includes a quantitation standard with values assigned in cp/μL. The latter producer has established conversion factor from cp/mL to IU/mL, but only for detection of hCMV DNA in EDTA plasma using specific amplification platform in combination with automated sample preparation and specific assay setup platform. Consequently, this conversion factor cannot be used generally. Moreover, a wide range of conversion factors (cp/mL to IU/mL) for the 1st WHO International Standard for Human Cytomegalovirus was found in a study of 11 laboratories participating in the Italian Infections and Transplant Working Group using dilution series of the IS in whole blood and plasma. The conversion factors calculated for the whole blood ranged from 0.21 to 1.17 and for plasma from 0.39 to 2.2 [38]. However generally the cp/mL to IU/mL conversion factor is close to 1.0 [8], which is consistent with the results of our study mentioned above, with conversion factor 1.12 for human plasma [37].

With a dPCR approach, absolute count of target sequences is possible, and the results are determined in copies per unit of volume. In addition, the accuracy of dPCR is capable of primary SI-traceability when measuring DNA in aqueous solution [39]. To further support standardization in nucleic acid amplification technique-based assays for hCMV multi-laboratory comparison studies are needed. This will allow laboratories to establish the correlation between IU and copies including consideration of pre-analytics. In addition, secondary standards could be better evaluated using dPCR as accurate secondary standards for viruses are lacking [40]. As reviewed in Hayden and Caliendo [41], dPCR has already been utilized as a reference standard method for measurements of commutability of reference materials and commutability of different assays. The importance of the assessment of dPCR for the improvement of the standardization of copy number assignment for secondary standards has been recognized in the Third International Consensus Guidelines on the Management of Cytomegalovirus in Solid-organ Transplantation [42].

In addition to serving as a reference measurement procedure, the dPCR method described here could serve clinical diagnostics directly. One factor influencing the high variability of results in routine hCMV load testing is amplicon size, with small amplicon sizes ($\leq$100 base pairs) tending to give higher viral loads compared to larger amplicons [43,44]. This may be particularly important when plasma is used for diagnostics, as in plasma the vast majority of hCMV DNA is cell-free (98–100%), highly fragmented, and not virion-associated [41,45], therefore the small amplicon of 72 bp utilized by our dPCR method is well suited to amplifying a higher proportion of the viral cell-free DNA present.

Introduction of this method to routine diagnostics or its use as a reference measurement procedure could support other aspects of standardization of routine hCMV testing. For example, there is still no consensus on the optimal biological specimen for testing as either plasma or whole blood are recommended [42] and better reliability and comparability of results could lead to selection of the optimal biological specimen. Similarly, as this method enables absolute quantification, it could support establishment of broadly applicable criteria for hCMV DNA thresholds or cut-off values that could be used in clinical decision making and are currently missing [38]. This could be achieved either in support of qPCR, via improved value assignment of calibration materials, or, as the method becomes more established, as direct method for clinical evaluation.

This work demonstrates that dPCR is well suited for the assignment of values of hCMV calibration and reference materials as well as for routine hCMV load testing. In EQA schemes, this method would allow the evaluation of hCMV concentration on the basis of reference measurement values instead of an evaluation on basis of consensus values mainly influenced by the market leaders.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank Helen Parkes (LGC) for guidance in the conception of the INFECTMET project and associated strategies for reference measurement procedure development.

Author statements

Concept and design of study MM, JFH, MKam, HPG, and HZ; Acquisition of data MM, JP, ABK, FVH, MKar, GMJ, JNee, and AP; Formal analysis and interpretation of data MM, JP, ABK, FVH, AP, MKar, GMJ, DOS, AD, MKam, HPG and HZ; Funding acquisition JFH, CAF, HS, MM, JNeu and AK; Drafting of the manuscript MM, ABK and AD; Review & editing: FVH, MKar, CAF, AP, AK, PC, JNeu, DOS, AD, MKam, HPG, HZ and JFH.

Funding

This study was financially supported by the INFECTMET project (the EMRP project; jointly funded by the EMRP participating countries within EURAMET and the European Union, project no. HLT08); AntiMicroResist project (which has received funding from the EMPIR program co-financed by the Participating States and from the European Union's Horizon 2020 research and innovation program, project no. 15HLT07); the Slovenian Research Agency (contract no. P4-0165 and 1000-13-0105); Ministry of Education, Science and Sport of republic of Slovenia (contract no. 3330-14-500133) and supported by the UK National Measurement System. The dPCR equipment used at NIB in this study was financed by the Metrology Institute of the Republic of Slovenia (MIRS), with financial support from the European Regional Development Fund.

The funding source(s) had no involvement in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. This publication reflects only the authors' s views and EURAMET is not responsible for any use that may be made of the information it contains.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2021.03.016.

References

[1] D.J. Speers, Clinical applications of molecular biology for infectious diseases, Clin. Biochem. Rev. 27 (2006) 39–51.
[2] M.J. Holden, R.M. Madej, P. Minor, L.V. Kalman, Molecular diagnostics: harmonization through reference materials, documentary standards and proficiency testing, Expert Rev. Mol. Diagnost. 11 (7) (2011) 741–755, https://doi.org/10.1586/erm.11.50.
[3] K. Messacar, S.K. Parker, J.K. Todd, S.R. Dominguez, C.S. Kraft, Implementation of rapid molecular infectious disease diagnostics: the role of diagnostic and antimicrobial stewardship, J. Clin. Microbiol. 55 (3) (2017) 715–723, https://doi.org/10.1128/JCM.02264-16.
[4] International Organization for Standardization, ISO 17822:2020 In vitro diagnostic test systems — Nucleic acid amplification-based examination procedures for detection and identification of microbial pathogens — Laboratory quality practice guide, (2020) 39.
[5] International Organization of Standardization, ISO 15189:2012 Medical laboratories — Requirements for quality and competence, (2012) 53.
[6] Bundesärztekammer, Instand e.V., Guidelines of the German Medical Association on quality assurance in medical laboratory testing, GMS Zeitschrift Zur Förderung Der Qual. Medizinischen Lab. 6 (2015) 43. http://www.egms.de/static/en/journals/lab/2015-6/lab000018.shtml.
[7] P. Hill, A. Uldall, P. Wilding, Fundamentals for External Quality Assessment (EQA), Ifcc. 31 (1996) 1–15. https://www.ifcc.org/ifcc-education-division/emd-committees/c-aq/c-aq-publications-and-resources/.
[8] W. Dimech, L.M. Cabuang, H.-P. Grunert, V. Lindig, V. James, B. Senechal, G. A. Vincini, H. Zeichhardt, Results of cytomegalovirus DNA viral loads expressed in copies per millilitre and international units per millilitre are equivalent, J. Virol. Methods 252 (2018) 15–23, https://doi.org/10.1016/j.jviromet.2017.11.001.
[9] H. Zeichhardt, O. Donoso Mantke, INSTAND Report External Quality Assessment Scheme Group 365 Virus Genome Detection – Cytomegalovirus September 2014, INSTAND e.V., Düsseldorf, 2014. https://www.instand-ev.de/uploads/tx_nfextinstandpdf/365_EN_Cytomegalovirus_Genome_EQAS_September_2014_20141116.pdf. [Accessed 11 December 2019].
[10] H. Zeichhardt, O. Donoso Mantke, INSTAND Report External Quality Assessment Scheme Group 365 Virus Genome Detection – Cytomegalovirus November/ December 2014, INSTAND e.V., Düsseldorf, 2015. https://www.instand-ev.de/uploads/tx_nfextinstandpdf/365_EN_Cytomegalovirus_Genome_November_December_2014_20150224.pdf. [Accessed 11 December 2019].
[11] H. Zeichhardt, M. Kammel, INSTAND Report on External Quality Assessment Scheme Group No. 365 Virus Genome Detection – Cytomegalovirus March 2017, INSTAND e.V., Düsseldorf, 2017. https://www.instand-ev.de/System/rv-files/365 EN Cytomegalovirus Genome EQAS Maerz 2017 20170613.pdf. [Accessed 11 December 2019].
[12] H. Zeichhardt, M. Kammel, INSTAND Report on External Quality Assessment Scheme Group No. 365 Virus Genome Detection – Cytomegalovirus June 2017, INSTAND e.V., Düsseldorf, 2017. https://www.instand-ev.de/System/rv-files/365 EN Cytomegalovirus Genome EQAS June 2017 20170905.pdf. [Accessed 11 December 2019].
[13] H. Zeichhardt, M. Kammel, INSTAND Report on External Quality Assessment Scheme Group No. 365 Virus Genome Detection – Cytomegalovirus September 2017, INSTAND e.V., Düsseldorf, 2017. https://www.instand-ev.de/System/rv-files/365 EN Cytomegalovirus Genome EQAS September 2017 20171115.pdf. [Accessed 11 December 2019].
[14] H. Zeichhardt, M. Kammel, INSTAND Report on External Quality Assessment Scheme Group No. 365 Virus Genome Detection – Cytomegalovirus November / December 2017, INSTAND e.V., Düsseldorf, 2018. https://www.instand-ev.de/System/rv-files/365 EN Cytomegalovirus Genome EQAS November December 2017 20180202.pdf. [Accessed 11 December 2019].
[15] H. Zeichhardt, M. Kammel, INSTAND Report on External Quality Assessment Scheme Group No. 365 Virus Genome Detection – Cytomegalovirus March 2018, INSTAND e.V., Düsseldorf, 2018. https://www.instand-ev.de/System/rv-files/365 EN Cytomegalovirus Genome EQAS March 2018 20180523.pdf. [Accessed 11 December 2019].
[16] J.F. Fryer, A.B. Heath, P.D. Minor, H. Kessler, W. Rawlinson, G. Boivin, J. Preiksaitis, X.-L. Pang, C. Barranger, S. Alain, C. Bressollette-Bodin, K. Hamprecht, T. Grewing, P. Constantoulakis, V. Ghisetti, M.R. Capobianchi, I. Abbate, C. Olivo, T. Lazzarotto, F. Baldanti, N. Inoue, F. Müller, C. Corcoran, D. Hardie, J. Prieto, R. Schuurman, A. van Loon, S. Ho, D. Hillyard, R. Hodinka, M. Louise Landry, A. Caliendo, N. Lurain, L. Sung, M. Gulley, C. Atkinson, J. Bible, M. Guiver, A collaborative study to establish the 1st WHO International Standard for human cytomegalovirus for nucleic acid amplification technology, Biologicals. 44 (2016) 242–251. https://doi.org/10.1016/j.biologicals.2016.04.005.
[17] R.J. Haynes, M.C. Kline, B. Toman, C. Scott, P. Wallace, J.M. Butler, M.J. Holden, Standard Reference Material 2366 for Measurement of Human Cytomegalovirus DNA, J. Mol. Diagn. 15 (2) (2013) 177–185, https://doi.org/10.1016/j.jmoldx.2012.09.007.
[18] H. Zeichhardt, O. Donoso Mantke, INSTAND Report External Quality Assessment Scheme Group 365 Virus Genome Detection – Cytomegalovirus September 2015, INSTAND e.V., Düsseldorf, 2015. https://www.instand-ev.de/uploads/tx_nfextinstandpdf/365_EN_Cytomegalovirus_Genome_EQAS_September_2015_20151117c.pdf. [Accessed 15 January 2020].
[19] H. Zeichhardt, O. Donoso Mantke, INSTAND Report external quality assessment scheme, group 370, virus genome detection, Influenza A and B viruses, March/ April 2014, INSTAND e.V., Düsseldorf, 2014. https://www.instand-ev.de/uploads/tx_nfextinstandpdf/361_EN_Hepatitis_B_Virus_Genome_EQAS_March_April_2014_20140603.pdf. [Accessed 15 January 2020].
[20] H. Zeichhardt, O. Donoso Mantke, INSTAND Report on External Quality Assessment Scheme Group 361 Virus Genome Detection – Hepatitis B Virus September 2014, INSTAND e.V., Düsseldorf, 2014. https://www.instand-ev.de/uploads/tx_nfextinstandpdf/361_EN_Hepatitis_B_Virus_Genome_EQAS_September_2014_20141114.pdf. [Accessed 15 January 2020].
[21] J. Pavšič, J. Žel, M. Milavec, Assessment of the real-time PCR and different digital PCR platforms for DNA quantification, Anal. Bioanal. Chem. 408 (1) (2016) 107–121, https://doi.org/10.1007/s00216-015-9107-2.
[22] J. Pavšič, A. Devonshire, A. Blejec, C.A. Foy, F. Van Heuverswyn, G.M. Jones, H. Schimmel, J. Žel, J.F. Huggett, N. Redshaw, M. Karczmarczyk, E. Mozioğlu, S. Akyürek, Müslüm Akgöz, M. Milavec, Inter-laboratory assessment of different digital PCR platforms for quantification of human cytomegalovirus DNA, Anal. Bioanal. Chem. 409 (10) (2017) 2601–2614, https://doi.org/10.1007/s00216-017-0206-0.
[23] J.F. Huggett, A.S. Whale, W. De Spiegelaere, W. Trypsteen, A.A. Nour, Y.-K. Bae, V. Benes, D. Burke, M. Cleveland, P. Corbisier, A.S. Devonshire, L. Dong, D. Drandi, C. A. Foy, J.A. Garson, H.-J. He, J. Hellemans, M. Kubista, A. Lievens, M.G. Makrigiorgos, M. Milavec, R.D. Mueller, T. Nolan, D.M. O'Sullivan, M.W. Pfaffl, S. Rödiger, E.L. Romsos, G.L. Shipley, V. Taly, A. Untergasser, C.T. Wittwer, S.A. Bustin, J. Vandesompele, J.F. Huggett, The Digital MIQE Guidelines Update: Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020, Clin. Chem. 66 (2020) 1012–1029. https://doi.org/10.1093/clinchem/hvaa125.
[24] Bundesaerztekammer, Neufassung der Richtlinie der Bundesaerztekammer zur Qualitaetssicherung laboratoriumsmedizinischer Untersuchungen – Rili-BAEK, Dtsch Arztebl. 116 (2019) A-2422 / B-1990 / C-1930. https://doi.org/10.3238/arztebl.2019.rili_baek_QS_Labor20192312.
[25] International Organization for Standardization, ISO 13528:2015 Statistical methods for use in proficiency testing by interlaboratory comparison, (2015) 89.
[26] H. Zeichhardt, M. Kammel, INSTAND-EXTERNAL QUALITY ASSESSMENT SCHEMES MANUAL Instructions for Test Performance Virus Immunology and Virus Genome Detection June 2017, INSTAND e.V., Düsseldorf, 2017. https://www.instand-ev.de/System/rv-files/2_Manual EQAS Virology June 2017 EN 20170602.pdf. [Accessed 11 December 2019].
[27] H. Zeichhardt, M. Kammel, INSTAND-EXTERNAL QUALITY ASSESSMENT SCHEMES MANUAL Instructions for Test Performance Virus Immunology and Virus Genome Detection September 2017, INSTAND e.V., Düsseldorf, 2017. https://www.instand-ev.de/System/rv-files/3_Manual EQAS Virology September 2017.pdf. [Accessed 11 December 2019].
[28] H. Zeichhardt, M. Kammel, INSTAND-EXTERNAL QUALITY ASSESSMENT SCHEMES MANUAL Instructions for Test Performance Virus Immunology and Virus Genome Detection November / December 2017, INSTAND e.V., Düsseldorf, 2017. https://www.instand-ev.de/System/rv-files/Manual EQAS Virology November December 2017 EN 20171106.pdf. [Accessed 11 December 2019].
[29] H. Zeichhardt, M. Kammel, INSTAND Report on External Quality Assessment Scheme Group No. 368 Virus Genome Detection – Cytomegalovirus – Training Program March 2017, INSTAND e.V., Düsseldorf, 2018. https://www.instand-ev.de/System/rv-files/368 EN Cytomegalovirus Training Genome March 2017 20180208.pdf. [Accessed 11 December 2019].
[30] H. Zeichhardt, M. Kammel, INSTAND-EXTERNAL QUALITY ASSESSMENT SCHEMES MANUAL Instructions for Test Performance Virus Immunology and Virus Genome Detection March 2018, INSTAND e.V., Düsseldorf, 2018. https://www.instand-ev.de/System/rv-files/Manual EQAS Virology March 2018 EN 20180214.pdf. [Accessed 11 December 2019].
[31] H. Zeichhardt, M. Kammel, INSTAND Report on External Quality Assessment Scheme Group No. 368 Virus Genome Detection – Cytomegalovirus – Training Program March 2018, INSTAND e.V., Düsseldorf, 2019. https://www.instand-ev.de/System/rv-files/368 EN Cytomegalovirus Training Genome March 2018 20190118.pdf. [Accessed 11 December 2019].
[32] H. Zeichhardt, O. Donoso Mantke, INSTAND-EXTERNAL QUALITY ASSESSMENT SCHEMES MANUAL Instructions for Test Performance Virus Immunology and Virus Genome Detection September 2014, INSTAND e.V., Düsseldorf, 2014. https://www.instand-ev.de/uploads/tx_nfextinstandpdf/Manual_EQAS_Virology_Sept_01.pdf. [Accessed 11 December 2019].
[33] H. Zeichhardt, O. Donoso Mantke, INSTAND-EXTERNAL QUALITY ASSESSMENT SCHEMES MANUAL Instructions for Test Performance Virus Immunology and Virus Genome Detection November / December 2014, INSTAND e.V., Düsseldorf, 2014. https://www.instand-ev.de/uploads/tx_nfext instandpdf/Manual_EQAS_Virology_November_engl_01.pdf. [Accessed 11 December 2019].
[34] H. Zeichhardt, O. Donoso Mantke, INSTAND Report External Quality Assessment Scheme Group 368 Virus Genome Detection – Cytomegalovirus – Training Program September 2014, INSTAND e.V., Düsseldorf, 2015. https://www.instand-ev.de/uploads/tx_nfextinstandpdf/368_EN_Cytomegalovirus_Training_Genome_September_2014_20150303.pdf. [Accessed 11 December 2019].
[35] H. Zeichhardt, M. Kammel, INSTAND-EXTERNAL QUALITY ASSESSMENT SCHEMES Instructions for Test Performance Virus Immunology and Virus Genome Detection March 2017, INSTAND e.V., Düsseldorf, 2017. https://www.instand-ev.de/System/rv-files/Manual EQAS Virology March 2017 EN 20170213.pdf. [Accessed 11 December 2019].
[36] J. Sassenscheidt, J. Rohayem, T. Illmer, D. Bandt, Detection of β-herpesviruses in allogenic stem cell recipients by quantitative real-time PCR, J. Virol. Methods 138 (1-2) (2006) 40–48, https://doi.org/10.1016/j.jviromet.2006.07.015.

[37] J. Pavšič, J. Žel, M. Milavec, Digital PCR for direct quantification of viruses without DNA extraction, Anal. Bioanal. Chem. 408 (1) (2016) 67–75, https://doi.org/10.1007/s00216-015-9109-0.

[38] F. Sidoti, A. Piralla, C. Costa, M.L. Scarasciulli, A. Calvario, P.G. Conaldi, P. Paba, C.F. Perno, A. Gaeta, G. Antonelli, G. Sodano, R. Santangelo, M. Sanguinetti, M. L. Vatteroni, L. Barzon, G. Palù, I. Abbate, M.R. Capobianchi, G. Piccirilli, T. Lazzarotto, F. Baldanti, R. Cavallo, Collaborative national multicenter for the identification of conversion factors from copies/mL to international units/mL for the normalization of HCMV DNA load, Diagn. Microbiol. Infect. Dis. 95 (2) (2019) 152–158, https://doi.org/10.1016/j.diagmicrobio.2019.05.012.

[39] H.-B. Yoo, S.-R. Park, L. Dong, J. Wang, Z. Sui, J. Pavšič, M. Milavec, M. Akgoz, E. Mozioğlu, P. Corbisier, Mátrai Janka, B. Cosme, J.J. de V. Cavalcante, R. B. Flatshart, D. Burke, M. Forbes-Smith, J. McLaughlin, K. Emslie, A.S. Whale, J. F. Huggett, H. Parkes, M.C. Kline, J.L. Harenza, P.M. Vallone, International Comparison of Enumeration-Based Quantification of DNA Copy-Concentration Using Flow Cytometric Counting and Digital Polymerase Chain Reaction, Anal. Chem. 88 (24) (2016) 12169–12176, https://doi.org/10.1021/acs.analchem.6b03076.s001.

[40] L. Tang, Y. Su, Z. Gu, A.M. Caliendo, S. Pounds, R.T. Hayden, A Comprehensive Statistical Framework for Determination of Commutability, Accuracy, and Agreement in Clinical DNAemia Assays, J. Clin. Microbiol. 57 (2018) 1–12. https://doi.org/10.1128/JCM.00963-18.

[41] R.T. Hayden, A.M. Caliendo, Persistent Challenges of Inter-assay Variability in Transplant Viral Load Testing, J. Clin. Microbiol. (2020) 1–23. https://doi.org/10.1128/jcm.00782-20.

[42] C.N. Kotton, D. Kumar, A.M. Caliendo, S. Huprikar, S. Chou, L. Danziger-Isakov, A. Humar, The Third International Consensus Guidelines on the Management of Cytomegalovirus in Solid-organ Transplantation, 2018. https://doi.org/10.1097/TP.0000000000002191.

[43] J.K. Preiksaitis, R.T. Hayden, Y. Tong, X.L. Pang, J.F. Fryer, A.B. Heath, L. Cook, A. K. Petrich, B. Yu, A.M. Caliendo, Are we there yet? Impact of the first international standard for cytomegalovirus DNA on the harmonization of results reported on plasma samples, Clin. Infect. Dis. 63 (5) (2016) 583–589, https://doi.org/10.1093/cid/ciw370.

[44] K. Naegele, I. Lautenschlager, R. Gosert, R. Loginov, K. Bir, I. Helanterä, S. Schaub, N. Khanna, H.H. Hirsch, Cytomegalovirus sequence variability, amplicon length, and DNase-sensitive non-encapsidated genomes are obstacles to standardization and commutability of plasma viral load results, J. Clin. Virol. 104 (2018) 39–47, https://doi.org/10.1016/j.jcv.2018.04.013.

[45] Y. Tong, X.L. Pang, C. Mabilangan, J.K. Preiksaitis, Determination of the biological form of human cytomegalovirus DNA in the plasma of solid-organ transplant recipients, J. Infect. Dis. 215 (2017) 1094–1101. https://doi.org/10.1093/infdis/jix069.