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Digital PCR method for detection and quantification of specific antimicrobial drug-resistance mutations in human cytomegalovirus

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ABSTRACT

Antimicrobial drug resistance is one of the biggest threats to human health worldwide. Timely detection and quantification of infectious agents and their susceptibility to antimicrobial drugs are crucial for efficient management of resistance to antiviral drugs. In clinical settings, viral drug resistance is most often associated with prolonged treatment of chronic infections, and assessed by genotyping methods; e.g., sequencing and PCR. These approaches have limitations: sequencing can be expensive and does not provide quantification; and qPCR quantification is hampered by a lack of reference materials for standard curves. In recent years, digital PCR has been introduced, which provides absolute quantification without the need for reference materials for standard curves. Using digital PCR, we have developed a rapid, sensitive and accurate method for genotyping and quantification of the most prevalent mutations that cause human cytomegalovirus resistance to ganciclovir.

1. Introduction

Antimicrobial drug resistance is acknowledged as one of the most prominent potential threats to human health (World Health Organization, 2018). In chronic infections, antimicrobial resistance arises from long-term treatment of infectious agents, which are often viral. Resistance can present as either persistent or increasing viremia or disease despite antiviral therapy. This can lead to serious consequences in an infected patient, as second-line antivirals might be more toxic or not available, which can lead to severe disease and even death (Strasfeld and Chou, 2010). For efficient management of resistance to antiviral drugs, timely detection and quantification of the infectious agent and its susceptibility to antimicrobial drugs are crucial, along with an understanding of the host factors, optimization of the drug delivery, knowledge of the mechanisms of resistance, and development of new antivirals (Hakki and Chou, 2011; Strasfeld and Chou, 2010).

The susceptibility of a virus to an antiviral drug is evaluated using both phenotypic and genotypic methods. As phenotypic methods are too time consuming for clinical diagnostic purposes, the need for rapid diagnosis has led to the development of genotyping methods; e.g., DNA sequencing and polymerase chain reaction (PCR). Sanger sequencing is the standard genotyping method for many viruses; nonetheless, it has many technical limitations. These include slow turn-around time and the complexity of the analysis, and above all the fact that mutations at < 20 % cannot be detected in this way (Chemaly et al., 2019). Newer, deep-sequencing technologies have overcome this issue, but these are limited by the lack of standardization and the high price (Chemaly et al., 2019). On the other hand, by sequencing part of the genome, previously unknown mutations can be detected. The advantage of PCR is the quick turn-around time, and in the case of real-time quantitative PCR (qPCR), quantification of the viral load is possible, which cannot be achieved by Sanger sequencing. However, quantification of point mutations with qPCR is limited, predominantly due to the lack of reference materials even for the most common mutations.

An alternative to the established genotyping methods is digital PCR (dPCR), which is a powerful tool for rapid and sensitive nucleic-acid quantification. Although the basic chemistry is the same as for qPCR, the quantification approach of dPCR is more advanced. Instead of relying on the relative quantification according to a standard curve, dPCR provides absolute quantification due to the binary nature of the

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reaction. While qPCR is performed in one tube, for dPCR the reaction is distributed across a large number of partitions that contain none, one or more copies of the target nucleic acid. After end-point PCR amplification, each partition is examined and defined as positive (i.e., presence of the PCR product) or negative (i.e., absence of the PCR product), hence the term 'digital'. The absolute number of target nucleic-acid molecules contained in the original sample before partitioning can be calculated directly from the fraction of positive versus total partitions, using binomial Poisson statistics (Pinheiro et al., 2012).

The independence of dPCR from the need for a standard curve is a major benefit, as this simplifies quantification of the target DNA or RNA sequences, and eliminates potential bias that can be introduced due to use of different materials between different laboratories (Baker, 2012). In addition, dPCR is more resistant to PCR inhibitors. Quantification remains possible with dPCR as long as the partitions with partial inhibition of amplification can be distinguished from the negative partitions, while in qPCR, the inhibition shifts the quantitation cycle or cq to a higher cycle, which leads to underestimation of the target concentration (Dingle et al., 2013; Rački et al., 2014). Several studies have shown that dPCR provides more robust, repeatable and precise nucleic-acid quantification compared to qPCR (Huggett et al., 2015, 2013; Pavšič et al., 2017; Yoo et al., 2016). dPCR has already been used for different applications in microbiology, such as quantification of viruses and bacteria (Devonshire et al., 2016; Hayden et al., 2016; Giovannelli et al., 2016; Pavšič et al., 2016a; Ricchi et al., 2017), value assignments of whole-virus reference materials (Bateman et al., 2017; Hayden et al., 2015; Pavšič et al., 2016b) and of certified plasmid DNA reference materials (Haynes et al., 2013; Sui et al., 2016), and detection of mutations that can confer antimicrobial drug resistance (Hennebique et al., 2017; Pholwat et al., 2013; Whale et al., 2016).

Human cytomegalovirus (HCMV), or human herpesvirus 5 (HHV-5), belongs to the Betaherpesvirinae subfamily, and like other herpes viruses, it can establish latent infections that can result in later recurrence of viral replication (Seitz, 2010). In healthy, immunocompetent individuals, HCMV infections show low pathogenicity and cause only mild symptoms, or even show no symptoms at all. However, in individuals with suppressed, compromised or immature immune systems, such as transplant recipients, patients with acquired immunodeficiency disease syndrome, or new-born babies, severe HCMV illness can occur (Chevillotte et al., 2010; Seitz, 2010). In 1988, ganciclovir became the first drug to be approved for the treatment of HCMV, and to date it remains the first-line treatment for HCMV infections (Gilbert and Boivin, 2005).

Herein, we studied the potential use of dPCR for detection of drug-resistant mutations of HCMV. We developed three methods that target mutations on codons 460, 594 and 595, which represent 70 % of the ganciclovir-resistant clinical isolates (Boivin et al., 2001). These three codons are part of the *UL97* gene, which codes for the viral protein kinase that can phosphorylate ganciclovir into its active form of ganciclovir monophosphate. Mutations in this gene thus prevent phosphorylation of the pro-drug, providing drug resistance to the viral strains. We tested the developed dPCR methods for specificity, repeatability and robustness, and then tested their applicability in external quality assessment schemes.

2. Materials and methods

2.1. Synthetic DNA design

Three different synthetic DNA sequences were used for primer and probe development and assessment. The synthetic DNA sequences were designed as double-stranded DNA fragments in the form of gBlocks (Integrated DNA Technologies, Coralville, Iowa, USA). They were designed as chimeric sequences between a fragment of a UL54 region that contained the wild-type sequence detected by a well-characterised method described by Sassenscheidt et al. (2006)), and further developed and evaluated by Pavšič et al. (2017). This method was used as reference method in this study (Table B1). The second fragment was the UL97 region containing ganciclovir resistance mutations or the wild-type sequence. The first of these chimeric sequences consisted of a part of UL54 and a part of UL97, that contained the ganciclovir resistance mutations M460 V and A594 V (DNA1; Data C1), the second sequence consisted of a part of UL54 and a part of UL97 that contained ganciclovir resistance mutation L595S (DNA2; Data C2), and the third sequence consisted of a part of UL54 sequence and a part of UL97 that contained the wild-type sequence (DNA3; Data C3) (Fig. A1). All of the gBlocks were shipped lyophilised and were reconstituted in TE buffer to a final concentration of 10 ng/μL.

2.2. Selection and design of the genotyping methods

The method that targeted the M460 V mutation was designed in-house using the Primer Express software, version 2.0 (Applied Biosystems). The methods that targeted A594 V and L595S were based on published methods (Volfova et al., 2014), with two adaptations: (1) for both A594 V and L595S, the same set of forward and reverse primers was used; and (2) the reverse primer was elongated by one additional nucleotide. All of the probes were TaqMan minor groove binder (MGB) marked with 6-carboxyfluorescein (FAM; Table B1). All of the primers and probes were from Eurofins MWG Operon (Ebersberg, Germany) or from Applied Biosystems (Inchinnan, UK). The primers and probes were shipped lyophilised and upon receipt, they were reconstituted in nuclease-free and protease-free water (Sigma-Aldrich Chemie GmbH, Munich, Germany).

2.3. In-silico specificity testing

First, the designed and selected oligonucleotides were tested for formation of hairpins and autodimers, and for specificity. The possible formation of hairpins was tested using UNAfold (Markham and Zuker, 2008). Oligo dimers or interactions between primers and probes were tested using Autodimer (Vallone and Butler, 2004). Autodimer makes ungapped local alignments of all of the primers and probes, giving +1 as a reward for a match, and −1 as a penalty for a mismatch. As recommended, a score of 7 was considered as a significant interaction. The *in-silico* specificity was tested by alignment of the primers to a nucleotide (nr) sequence database in Primer-BLAST (Ye et al., 2012). Two searches were made: first, the primer sequences were blasted against the whole nr database, and the second search was performed on the nr database excluding viral sequences (as it is not possible to exclude a single taxonomic entity from the search, only the following organisms were included human, animal, plant, bacteria and fungi). Default parameters were used, with one exception: the minimum length of the PCR product was changed to the combined length of both primers (for A594 V and L595S, 39 nucleotides; for M460 V, 42 nucleotides).

2.4. Digital PCR and data analysis

All dPCR experiments were performed using the QX100/QX200 Droplet Digital PCR (ddPCR) system (Bio-Rad). The 20 μL reaction was prepared by mixing 10 μL 2 × ddPCR Supermix for probes (no dUTP) (Bio-Rad, Pleasanton, CA, USA), 6 μL of the corresponding primers and probe (for final concentration see Table B1), and 4 μL DNA template (or 4 μL nuclease-free and protease-free water for non-template controls). The reaction mixture was pipetted into the sample well of a QX100 droplet generator system (Bio-Rad) DG8 cartridge, where the droplets were generated. The emulsion was then transferred into 96-well plates and amplified using a C1000 or T100 Touch Thermal cycler (Bio-Rad). Thermocycling conditions followed a touchdown approach, and were 95 °C for 10 min, 15 cycles of 94 °C for 30 s, and gradually decreasing annealing temperature from 70 °C to 58 °C over 1 min, 30 cycles of 94 °C for 30 s, and 58 °C for 1 min, followed by 98 °C for 10 min, and a 4 °C

hold. The ramp rate for each step was set to 2 °C/s. Droplets were read using either QX100 or QX200 Droplet Digital System droplet reader (Bio-Rad). Data acquisition and analysis was performed using QuantaSoft software, version 1.7.4.0917 (Bio-Rad). Thresholds were set for each method (at amplitude approximately 3300, 3000 and 1300 for the methods targeting A594 V, L595S and M460 V, respectively, and for the reference method at amplitude approximately 3000) using fluorescence amplitude versus event number (i.e., one-dimensional amplitude), to define the positive and negative droplets. The data were exported as comma-separated values files and further analysed using Microsoft Excel spreadsheets. The data generated by the QX100 or QX200 droplet reader were rejected from subsequent analysis if a clog was detected by the Quantasoft software or if a low number of droplets (< 10,000) was measured per 20 µL PCR. All dPCR experiments were implemented according to the dMIQE guidelines (Table B2).

2.5. Real-time quantitative PCR

All qPCR experiments were performed in two technical replicates using either on Viia™ 7 Real–Time PCR System or QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems). The 10 µL reaction was prepared by mixing 2.5 µL TaqMan Universal PCR Master Mix (Applied Biosystems), 4.5 µL of the corresponding primers and probe (for final concentration see Table B1), 1 µL of nuclease-free and protease-free water and 2 µL DNA template (or 2 µL nuclease-free and protease-free water for non-template controls). The following cycling conditions were used 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, using standard temperature ramping mode. The primer and probe sequences and the concentrations in the final reactions were the same as for the dPCR (Table B1).

2.6. Synthetic DNA characterization

Each of the three synthetic DNA fragments was first characterized in terms of target copies per µL, using the reference method. For this purpose, six gravimetric dilutions were prepared (1:100, 1:10,000, 1:800,000, 1:1,000,000, 1:1,200,000 and 1:120,000,000; Fig. A2). The concentration was assigned using dPCR and the reference method, on the last four dilutions of the series. These dilutions were tested on two consecutive days as six technical replicates (three technical replicates per day). The reaction mixture was also prepared gravimetrically. The results of the two experiments were combined and the concentrations of the first two dilutions and the stock solutions were calculated (Table B3).

2.7. Dynamic range, repeatability, and limits of detection and quantification

Upon characterization of synthetic DNA fragments, two dilution series were prepared, in terms of target copy numbers. First, the initial dilution (dilution 1) was prepared that targeted either 6000 or 18,750 copies per reaction for DNA1 and DNA2, from which a dilution series was prepared (referred to as dilutions 2–8) representing 1:2, 1:6, 1:60, 1:120, 1:240, 1:720, 1:2,160 dilutions for DNA1 and 1:10, 1:30, 1:300, 1:600, 1:1,200, 1:3,600, 1:18,000 dilutions for DNA2. The dilutions were prepared in bulk, aliquoted in DNA low bind tubes and stored at below −20 °C. One of the dilutions of each dilution series was measured with the reference method (3 days; each day, three technical replicates), and the means were used to assign the copy numbers for all of the other dilutions. The final assigned values of the dilution series were from 5600 to 3 copies per reaction for DNA1, and 15,300 to 1 copy per reaction for DNA2 (Table 1). Fifteen replicates of the dilution series were measured by dPCR (three separate runs, over 3 days, each containing five technical replicates). Before determination of the limit of quantification (LOQ) and detection (LOD), potential outliers were removed from the dataset (Table B4). First, the third (Q3) and first (Q1) quartile, and inner quartile range (IQR) were calculated using Microsoft Excel. To identify potential outliers, Eqs. (1) and (2) were used.

$$outlier < Q1 - 1.5 * IQR \quad (1)$$

$$outlier > Q3 + 1.5 * IQR \quad (2)$$

The LOQ and LOD for the dPCR were determined based on these experimental data, and so was the repeatability and the dynamic range. The LOQ was determined as the lowest concentration in the dilution series that consistently yielded a relative standard deviation (RSD) < 25 %. The LOD was determined as the lowest concentration in the dilution series that consistently yielded positive signals with all replicates.

Table 1
Limits of detection and quantification for the three dPCR methods.

Dilution [a]	M460V		A594V		L595S	
	CV(%)[b]	Mean (cp/rnx)	CV(%)[b]	Mean (cp/rnx)	CV(%)[b]	Mean (cp/rnx)
1	6396	3.5	7035	4.2	15,891	13.9
2	2752	2.9	3029	4.6	1183	8.7
3	973	15.7	1034	19.1	373	15.1
4	76[c]	14.3	82	18.5	37[c]	16.4
5	37	29.6	33	24.4	**17**[d]	**37.8**
6	**20**[d]	**35.1**	**19**[c,d]	**20.1**	8[e]	ND
7	6[e]	ND[f]	5[e]	ND[f]	1[e]	ND
8	4[e]	ND[f]	4[e]	ND[f]	ND[f]	ND[f]

[a] For A460 V and A594 V, the dilutions series was 5600, 2800, 933, 93, 47, 23, 8 and 3 copy numbers/reaction (cp/rnx), and for L595S it was 15,300, 1530, 510, 51, 26, 13, 3 and 1 cp/rnx. [b] CV% was calculated for all technical replicates across the three experiments. [c] LOQ according to total cp/rnx. [d] LOD according to total cp/rnx. [e] At least one replicate was negative. [f] Not determined due to negative replicate(s). ND, not determined. Bold: limit of detection; underlined: limit of quantification.

2.8. Robustness

The robustness of the methods was evaluated by comparison of the original method to two modifications: (1) 20 % increase in the primer and probe concentrations in the reaction; and (2) 20 % decrease in the primer and probe concentrations in the reaction. Two samples from the repeatability experiments (dilution 4, dilution 6) were tested in five technical replicates in the robustness experiments.

2.9. Applicability

Nine samples (349005, 349006, 349007, 349008, 349009, 349010, 349011, 349012, 349016) from INSTAND external quality assessment (EQA) scheme 'Virus Genome Detection - Cytomegalovirus Resistance Determination' (group number 349) terms 2016, 2017 and 2018 were tested to determine the applicability of the methods. The samples comprised cell culture supernatants of viral cultures from isolates derived from urine or blood of HCMV-positive donors diluted using HCMV-negative plasma. Each sample arrived in four vials. Viral DNA was extracted from two vials for each sample using High Pure viral nucleic acid kits (Roche) in two extraction replicates. For the first four samples (EQA scheme term 2016) one extraction replicate was chosen and tested with qPCR for the presence of the virus (using the reference method) and the M460 V and A594 V mutations. For the positive samples, each extraction replicate was tested in two technical replicates using dPCR, which resulted in eight measurements per sample. The results are presented as fractional abundance of the mutation compared to the whole HCMV. Mutation % was calculated using Eq. (3). For the EQA scheme terms 2017 and 2018, no qPCR pre-run was performed, and all of the samples were immediately tested on dPCR; each extraction was repeated as two technical replicates.

$$mutation\ \% = \frac{cp/rnx\ mutation}{cp/rnx\ HCMV} * 100 \quad (3)$$

3. Results

3.1. Selection of the model system and in-silico specificity assessment

To create a representative system that includes the mutations on each of the 460, 594 and 595 codons, we selected the following three specific mutations: M460 V, A594 V and L595S. The primer and probe pair used for the method that targets the M460 V mutation was developed in-house, while those for the methods that target the A594 V and L595S mutations were modified from Volfova et al.(Volfova et al., 2014) (Table B1).

In the first step of the analysis, the specificities of the methods were tested *in-silico* using Primer-BLAST (Ye et al., 2012). There were no unintended amplicons found for the methods that targeted A594 V and L595S, but there were two found for the method that targeted M460 V (Data C4). However, further analysis of these two unintended amplicon sequences showed that they did not contain the probe sequence, and thus did not interfere with target quantification. We also showed that the primer and probe sequences did not form any hairpins and loops (UNAFold (Markham and Zuker, 2008)), and that there were no interactions between the pairs of oligonucleotides, the primers and the probe (AutoDimer (Vallone and Butler, 2004)).

3.2. Assessment of the method

Six gravimetric dilutions of synthetic DNA were prepared (Fig. A2), which were tested as six technical replicates over two days on the QX100/QX200 platforms, and assigned the copy numbers of 4.6×10^9, 4.4×10^9 and 5.5×10^9 for DNA1, DNA2 and DNA3, respectively (Table B3).

In the preliminary analysis, the detection of each of these three mutations, M460 V, A594 V and L595S, using qPCR was also addressed, as in clinical settings qPCR is frequently used and is at present still faster and less expensive compared to dPCR. Using qPCR all three methods showed at least a 10-fold difference when compared to the reference method (Fig. A3) and would need extensive optimisation. As shown by Pavšič et al. (Pavšič et al., 2016a) several fold differences in qPCR can be significantly decreased in dPCR. Based on this, only dPCR has been chosen for further optimisation.

In the initial optimisations of dPCR different annealing temperatures and touchdown PCR were tested to obtain optimal separation of negative and positive droplets. Based on the performance, touchdown approach with the thermocycling conditions described in the Materials and methods (2.4 Digital PCR and Data Analysis) has been selected.

3.2.1. In vitro specificity assessment

To confirm the specificity of the dPCR methods *in vitro*, all three methods were tested against the wild-type (DNA3, Data C3). This showed some cross-reactivity, as is often the case in the detection of point mutations. In dPCR cross-reactivity is presented in the form of an additional cluster between the true positive and the negative cluster. In our case, the additional cluster was very close to the negative cluster for all three of the methods (Fig. A4). Consequently, some of the true-positive partitions are defined as negative. Therefore, we can set two thresholds: the first just above the negative cluster (Fig. 1, full line); and second above the false-positive cluster (Fig. 1, dashed line). The impact of these two thresholds on the copy number counts can then be assessed. The bias of the copy number counts between thresholds was 2 % for the M460 V and A594 V methods, and 9 % for the L595S method. We finally chose fixed thresholds of 1300, 3000 and 3300 for methods M460 V, L595S and A594 V, respectively. These fixed thresholds were the highest of those tested, and were set above the false-positive clusters (Fig. A4, the threshold corresponds to the dashed pink line in Fig. 1), with the exclusion of the majority of false-positive partitions. These thresholds were imposed on all of the further analyses.

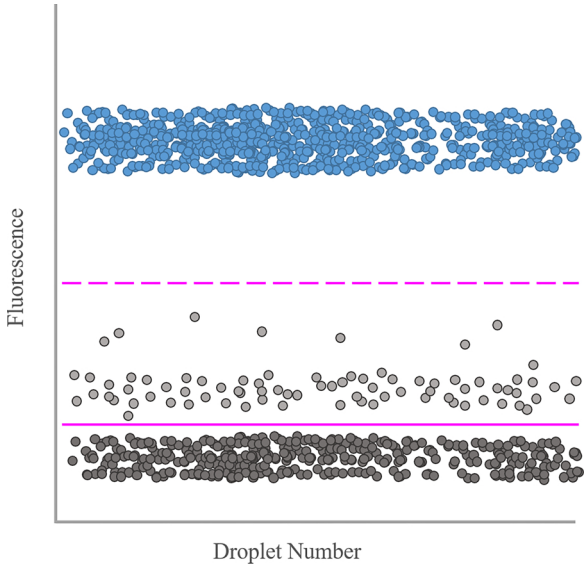

Fig. 1. The two different thresholds set for each of the dPCR methods. The lower threshold (full pink line) was set just above the negative cluster (dark grey), and the higher threshold (dashed pink line) was set above the false-positive cluster (light grey). Blue symbols represent the positive cluster. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.2.2. Estimation of the limits of detection and quantification

With the characterisation of the synthetic DNA fragments and determination of the appropriate fixed thresholds, we then continued to the assessment of the methods. Limits of detection (LODs) and quantification (LOQs), working range, precision (i.e., repeatability, intermediate precision), robustness and trueness were determined on a dilution series that comprised eight DNA dilutions (dilutions 1–8). The targeted copy numbers of these dilutions were calculated from the assigned copy numbers (Table B3). Fifteen replicates were tested for each of the three methods on three separate days. The dataset was reviewed for outliers. A total of six outliers were removed from the dataset (two for each method); however, these were not more than two per dilution (Table B4). Thus, at least 13 replicates were used for assessment of these methods.

The LOD was determined to be 20 copies/reaction (cp/rnx) for the M460 V method, 19 cp/rnx for the A594 V method, and 17 cp/rnx for the L595S method (Table 1; Table B5). The lowest copy number concentrations (i.e., cp/rnx) that yielded a positive signal were determined for each of the replicates for each of the experiments. A reaction was deemed positive if it had at least three positive partitions. The LOQs were determined as 76, 19 and 37 cp/rnx for the M460 V, A594 V and L595S methods, respectively (Table 1; Table B5). The LOQ was set at the lowest copy number concentration for which the coefficient of variability (CV) was consistently $\leq$ 25 % within each individual experiment. In the case of the A594 V method, the LOQ corresponded to the LOD.

The working range of the method was defined by the series of copy number concentrations within which the target can be reliably quantified with acceptable uncertainty. The lower limit of the working range was thus restricted by the LOQ, while the upper limit was restricted by the sample concentration used in the analysis. The working ranges were determined as from 76 cp/rnx to 6500 cp/rnx for the M460 V method, 19 cp/rnx to 7000 cp/rnx for the A594 V method, and 37 cp/rnx to 16,000 cp/rnx for the L595S method (Tables 1,2).

Table 2
Intermediate precision for the three methods.

Dilution [a]	M460V		A594V		L595S	
	Mean(cp/rnx)	CV(%)[b]	Mean(cp/rnx)	CV(%)[b]	Mean(cp/rnx)	CV(%)[b]
1	6396	3.1	7035	3.8	15,891	3.9
2	2752	1.2	3029	3.4	1183	5.7
3	973	18.3	1034	22.1	373	11.6
4	76	11.2	82	19.1	37	5.7
5	37	24.7	33	22.5	17	25.7
6	20	18.9	19	13.1	< LOD[c]	ND[d]
7	< LOD[c]	ND[d]	< LOD[c]	ND[d]	< LOD[c]	ND[d]
8	< LOD[c]	ND[d]	< LOD[c]	ND[d]	ND[d]	ND[d]

[a] For A460 V and A594 V, the dilutions series was 5600, 2800, 933, 93, 47, 23, 8 and 3 cp/rnx, and for L595S it was 15,300, 1530, 510, 51, 26, 13, 3 and 1 cp/rnx
[b] CV% between experiments/days; dilutions 1 − 8. [c] LOD according to cp/rnx. [d] ND, not determined due to negative replicate(s).

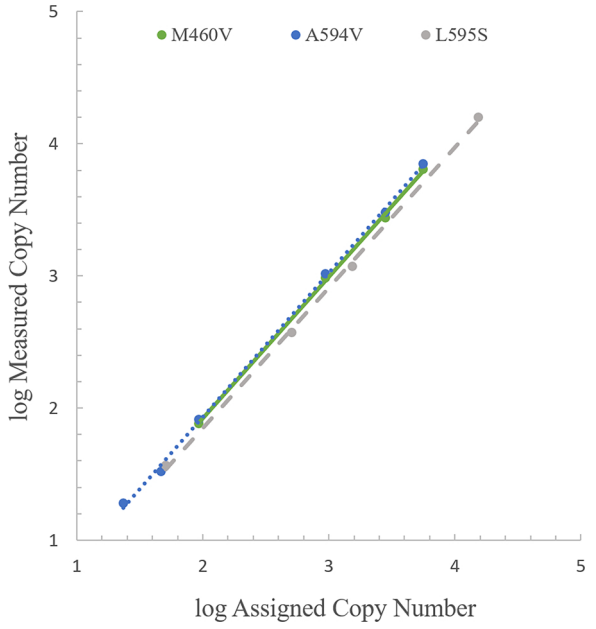

Fig. 2. Dynamic range of the dPCR methods. All of the methods showed high linearity (R2 > 0.998) for all of the concentrations above the estimated limit of quantification (R2: M460 V, 0.9991; A594 V, 0.999; L595S, 0.9989). Each data point represents the mean of three independent experiments, with four or five technical replicates.

Additional parameter of the dynamic range included the linearity and the intermediate precision, i.e. the variability between experiments conducted on different days. The linearity for all of the methods was > 0.998 (Fig. 2) and the CV between experiments was < 25 % for all of the dilutions at or above the LOQ for all of the methods (Table 2).

3.2.3. Robustness

To investigate the influence of small deliberate changes made to the method parameters, we tested the robustness of the methods by varying the concentration of the primers and probes used in the reaction mixture. Five technical replicates were tested for each change at two concentrations: one in the dynamic range (dilution 4), and one close to the LOD (dilution 6). All of the reactions at the concentration close to the LOD were positive, and all of the reactions at the concentration within the dynamic range had CV < 25 %, with the exception of the L595S methods, where the CV was a little above 25 %, at 27.5 % and 29.1 %, for the original primers and probe concentration and for the +20 % variation, respectively (Table 3).

3.2.4. Trueness

The trueness of the method is the closeness of agreement between the value measured by the method in question and the accepted reference value; i.e., the value assigned by the reference method. Trueness is usually expressed in terms of bias. As the material used for validation was a chimeric sequence, the copy number of the material could be assessed using two methods: the reference method, and the methods targeting the mutations. In this way, the closeness of agreement was assessed between the newly developed methods and the well-characterised reference method. The bias between the copy number concentration assigned by the reference method and the copy number concentration measured by the developed methods was calculated (Table B6). The bias was < 25 % in all cases for the M460 V method, but it was a little > 25 % for dilutions 1 and 5 for the A594 V method. For L595S the bias was between 3.86 % and 33.33 %.

3.3. Fitness for purpose

Samples from the EQA scheme terms 2016, 2017 and 2018 were tested to determine the fitness for purpose of the methods. Samples from EQA scheme term 2016 were initially screened by qPCR for the presence of HCMV and the mutations M460 V, A594 V. The qPCR prerun showed that sample 349008 did not contain the M460 V or the A594 V mutations (Table B7), whereas samples 349005 and 349006 contained mutation M460 V, and sample 349007 mutation A594 V (Table 4; Table B7).

Detection of the mutations using qPCR was followed by quantification with dPCR. The mean copy numbers per mL of plasma were calculated together with the CV. For all of the samples and all of the targets in all of the studies, the CVs were < 25 %, except for sample 349009, where the CV for both the reference method and the A594 V method was > 25 % (Table B8). The mutation % was calculated for each extraction parallel of two vials, which provided four data points for each sample (Appendix B Tables B9–B11), with the data expressed as means (Table 4). The CV between these individual results was < 25 %, except for sample 349006, for which the CV was a little above (27.12 %; Table B9).

Samples from the EQA scheme terms 2017 and 2018 were directly quantified using dPCR. For the EQA scheme term 2017, the CMV containing sample 349010 was negative for each of the above mentioned mutations. Samples 349009 and 349011 were correctly analysed positive for A594 V and sample 349012 was correctly analysed positive for M460 V. The CVs of the copy numbers were < 25 % for samples 349011 and 349012, but > 25 % for sample 349009. For the EQA scheme term 2018, sample 349,016 was analysed positive for the L595S mutation (Table B11). The CV of the copy numbers was < 25 % for both methods, the reference method and the method targeting the mutation (Table B8).

Finally, the mutation % for all EQA schemes were calculated as the means of the four data points of each of the samples (Table 4). The mutation CVs were < 25 % for all tested samples (Appendix B Tables B9–B11).

Table 3
Robustness of the methods.

Protocol	Dilution [a]	M460V			A594V			L595S		
		Mean (cp/rnx)	CV (%)	Bias to original (%)	Mean (cp/rnx)	CV (%)	Bias to original (%)	Mean (cp/rnx)	CV (%)	Bias to original (%)
Original	4	53	13.7	/	62	6.3	/	49	27.5	/
	6	16	18.6	/	18	35.3	/	9	41.9	/
+20 %	4	53	21.6	−0.1	58	15.4	−6.8	46	29.1	−5.3
	6	15	42.6	−7.6	15	14.5	−16.0	9	76.6	1.5
−20%	4	58	10.0	9.6	61	24.1	−2.3	47	12.9	−2.8
	6	17	26.3	2.2	17	7.4	−6.9	7	53.1	−26.2

[a] Dilution 4 is within dynamic range, dilution 6 is near LOD.

Table 4
Identification and content of the mutations in the samples from the external quality assessment scheme.

EQA scheme term	Matrix	Sample	Mutation detected	Mutation %
2016	Plasma	349005	M460V	27.16
		349006	M460V	34.53
		349007	A594V	67.18
		349008	Neg.[a]	ND[b]
2017	Plasma	349009[c]	A594V	41.56
		349010	Neg.[a]	ND[b]
		349011[c]	A594V	42.34
		349012	M460V	21.87
2018	Plasma	349016	L595S	87.71

[a] Neg., All replicates were negative. [b] ND, Not determined due to negative replicate(s). [c] Samples 349009 and 349011 are identical.

4. Discussion

In the present study, we have demonstrated the potential of dPCR for genotyping by developing three dPCR methods for detection and absolute quantification of the three most common mutations that confer resistance of HCMV to ganciclovir. These methods are more rapid and sensitive than those used today; e.g., Sanger or next-generation sequencing. Although these new methods are limited to detection of known mutations, they can be used to support detection of drug-resistant mutations in clinical settings, by indirectly underpinning the development and evaluation of sequencing approaches, or they can be used for value assignment of control or reference materials needed for PCR/qPCR or materials used in external quality assessment schemes.

For assessment of the methods, synthetic DNA was used as a chimeric construct that comprised the partial sequences of two HCMV genes: part of UL97, which codes for a protein kinase, as the wild-type or carrying the targeted mutations; and part of UL54, which codes for a viral DNA polymerase, as the wild-type only (Fig. A1). Three such synthetic DNA fragments were designed, where the first contained the M460 V and A594 V mutations (DNA1; Data C1), the second contained the L595S mutation (DNA2; Data C2), and the third represented the wild-type UL97 (DNA3; Data C3). For characterization of the synthetic DNA, a well-characterized method was used that targets UL54, which is from here on referred to as the reference method (Pavšič et al., 2017; Sassenscheidt et al., 2006).

The fundamental properties of a method are the ability to discriminate between the target and non-target (specificity) and the ability to amplify the target. We have shown the specificity of the developed methods both *in-silico* and *in-vitro*. Although *in-silico* analysis showed that our methods do not amplify any unintended targets, further *in-vitro* analysis showed some cross-reactivity to the wild-type sequence. However, we were able to differentiate between the wild-type and the mutated sequence using dPCR. Here the power of partitioning is clearly demonstrated. The cross-reactivity is not detected in qPCR as the fluorescent signal coming from the unintended amplification is added to the overall signal and one does not see the cross-reactivity. In dPCR, however, the effect of cross-reactivity is clearly seen and can be evaluated and reduced by finding an optimal temperature via gradient PCR, or where this approach fails, it can be minimised by a touch-down cycling protocol, as was done in this study. Since the cross-reactivity manifested itself in an additional cluster very close to the negative cluster it's effects were further minimised by the appropriate positioning of the threshold. The positioning of the thresholds is a part of the bias that the newly developed methods show in comparison to the reference method, which is discussed further below.

In the following steps, the methods were assessed for LOD and LOQ, robustness and trueness. With 20 cp/rnx or below the LOD was relatively low for all three methods. In addition, when we look at individual experiments (Table B5), we can see that there is a potential for even lower LOD, such as in the case of M460 V experiment 1, where LOD is determined at 7 cp/rnx. The LOQ was relatively high for the M460 V method (76 cp/rnx), compared to the other two methods, 19 and 37 cp/rnx for the A594 V and L595S, respectively. Looking closely at the data from the individual experiments (Table B5), it can be seen that for experiment #1, the CV for dilution 5 was > 25 %, whereas for experiments #2 and #3 the CV for dilution 5 was < 25 %, which suggests that the LOQ might be lower. Although a more precise determination of LOD and LOQ would be possible with a dilution series with more dilution points, the working range is still limited by the dPCR platform itself. The theoretical upper limit of the working range is ~200,000 cp/rnx for 20,000 partitions (Pecoraro et al., 2019). This means that the working range could be extended by testing samples that were more concentrated as the cp/rnx used were well below the upper limit of the QX100/200 working range. However, these additional tests were not necessary, as they would not have contributed to the overall assessment of the applicability of these methods.

The method is considered robust when the bias between copy number concentrations from the original and the varied conditions does not exceed ± 30 % in the dynamic range, and where all of the reactions are positive at the copy number concentration near to the LOD (Table 3). As the bias here was below ± 30 % even at the copy number concentrations near the LOD in all cases, these methods can be considered as extremely robust.

In the final step of the method assessment we determined the trueness of individual methods by comparing the cp/rnx to the reference method. The most critical was the L595S method, which consistently showed lower copy numbers than those assigned by the reference method. For the L595S method, the estimated underestimation of copy number concentrations due to the threshold setting was 9 %. When we enlarged the copy number of L595S by 9 %, the bias decreased by ~6 %, and was below the 25 % threshold (data not shown). When we applied the same correction for the M460 V and A594 V methods, we did not observe any changes in the data. This is not surprising, as the estimated underestimation was only 2% for the M460 V and A594 V methods, and as the bias to the reference method did not show any pattern (Table B6).

In order to give a proof of principle for the applicability of the developed methods, these methods were applied for testing quality

control samples in cooperation with INSTAND e.V., Düsseldorf, an interdisciplinary, not-for-profit, scientific medical society which serves as provider of international external quality assessment (EQA) schemes. The well-defined EQA schemes samples derived from the INSTAND EQA scheme "Virus Genome Detection - Cytomegalovirus Resistance Determination" (program 349) from the years 2016, 2017 and 2018. The samples contained CMV isolates carrying different ganciclovir resistance associated mutations in UL97 (M460 V, M460I, A594 V and L595S) (Table 4). The main emphasis of these EQA schemes have been the recognition of mentioned mutations by sequencing or other methods with subsequent interpretation of ganciclovir susceptibly.

For the evaluation of the samples from the EQA scheme term 2017, we have chosen to use both qPCR and dPCR method. However, even with additional optimization of the qPCR methods, their applicability for quantification of mutations would be limited due to the lack of the relevant reference materials. For this reason, we focused on dPCR, which is more suitable for quantification of mutations and for monitoring of the efficiency of a treatment. Nonetheless, qPCR is suitable for detection of all three of these mutations, in terms of rapid screening; hence its inclusion in this assessment of the applicability of these methods.

Our goal was not only to detect the individual mutations in EQA samples, but also to quantify the samples in terms of fractional abundance of mutation (mutation %), i.e the fraction of the mutation compared to the overall content of the virus. To achieve this, we used the reference method to determine the cp/mL of plasma of the whole HCMV, wild type and mutated, and the methods targeting the mutations to determine the cp/ mL of plasma of an individual mutation. The cp/ mL of plasma were then used to calculate the mutation %.

The correct mutations were determined for all of the samples in all three studies (INSTAND e.V., 2019, 2018, 2017), which shows that these methods are fit for purpose and give results that are comparable to other genotyping techniques. The samples designated 349009 and 349011 were identical. Both samples were correctly analysed positive for A594 V but the CV was observed to be high for both samples and both methods, the reference method and the method targeting the mutation, however mutation % was comparable (Table 4; Table B10) confirming the applicability of these methods for detection and quantification of selected mutations in these samples. Careful evaluation of the performance of newly developed methods including the comparison to established methods is crucial before their introduction into the laboratory. With this study, we have shown that dPCR has great potential in microbiology not only for genotyping and absolute pathogen quantification, but also for detection of drug-resistance mutations. As dPCR provides absolute quantification without the need for reference materials for standard curves, this method could be established as a reference method and as a method for evaluation and value assignment of materials for EQAs or newly developed control or reference materials to support other methods such as qPCR and sequencing. Although the methods for detection and quantification of three mutations were compared to the previously established method for HCMV detection and quantification further investigations including additional inter-laboratory studies will be necessary in order to show the strength of these newly developed methods for support in clinical virology. By developing methods for additional frequent mutations and testing their performance in clinical studies, these methods might also be directly applicable in routine laboratories to speed up detection and quantification of known mutations in HCMV enabling better management of the disease. Moreover, the principle of quantification shown in this study could be applied to any other infectious agent with known mutations conferring resistance.

5. Conclusions

In summary, we have developed three dPCR methods for detection and absolute quantification of the three most common mutations that confer resistance of HCMV to ganciclovir. These methods are more rapid and sensitive than those used today; e.g., Sanger or next-generation sequencing. Although, they are limited to detection of known mutations, they can be used to support detection of drug-resistant mutations in clinical settings, by indirectly underpinning the development and evaluation of sequencing approaches, or they can be used for value assignment of control or reference materials needed for PCR/qPCR or materials used in external quality assessment schemes. Following additional clinical studies, these methods might also be directly applicable to speed up detection of known mutations or allow detection in specimens with low level presence of virus, or specimens with fragmented DNA, where the use of next-generation sequencing is limited. We have also shown that careful evaluation of the performance of these methods is needed before their introduction into the laboratory, which will be better supported by comparisons of these newly developed methods with those already established.

Author contributions

Alexandra Bogožalec Košir conceived and planned the experiments, carried out the experiments, analyzed the data, and took the lead in writing the manuscript. Tašja Cvelbar carried out the experiments and partly analyzed the data, and contributed to the final version of the manuscript. Martin Kammel, Hans-Peter Grunert and Heinz Zeichhardt contributed to the supply of the samples of the INSTAND external quality assessment schemes and to the final version of the manuscript. Mojca Milavec conceived the study and was in charge of overall direction and planning, conceived and planned the experiments, and partly analyzed the data.

Author statement

Alexandra Bogožalec Košir conceived and planned the experiments, carried out the experiments, analysed the data, and took the lead in writing the manuscript.

Tašja Cvelbar carried out the experiments and partly analysed the data, and contributed to the final version of the manuscript.

Martin Kammel, Hans-Peter Grunert and Heinz Zeichhardt contributed to the supply of the samples of the INSTAND external quality assessment schemes and to the final version of the manuscript.

Mojca Milavec conceived the study and was in charge of overall direction and planning, conceived and planned the experiments, and partly analysed the data.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

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Appendix A

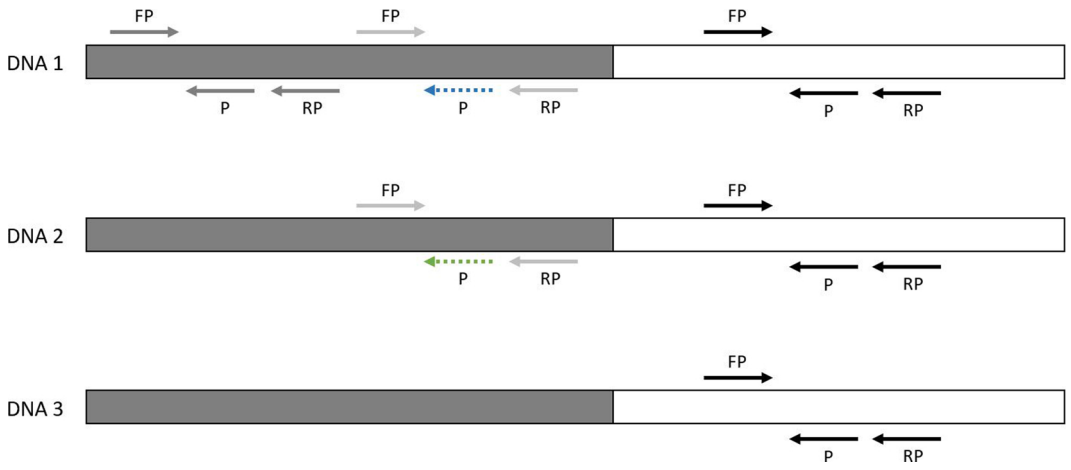

Fig. A1. Diagram of the chimeric structure of DNA1, DNA2 and DNA3. Gray areae corresponds to partial sequence of *UL97* and the white to *UL54*. Arrows denote primers (FP - forward primer, RP - reverse primer) and probes (P) and their orientation: dark grey corresponds to assay targeting A460 V, light grey correspond to primers of the assays targeting A594 V and L595S (primer sequences are the same), blue dashed (DNA1) corresponds to the probe for A594 V and green dashed (DNA 2) for L595S targeting assay, in black are primers and probes for the reference method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

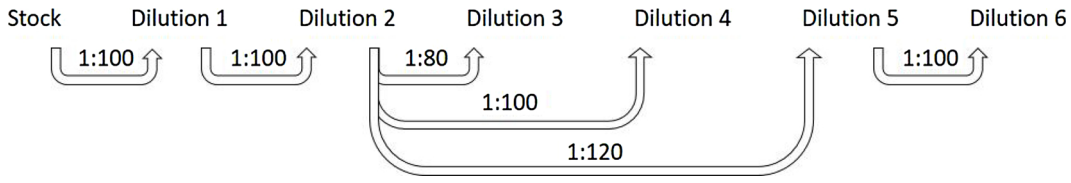

Fig. A2. Gravimetric dilutions of synthetic DNA fragments.

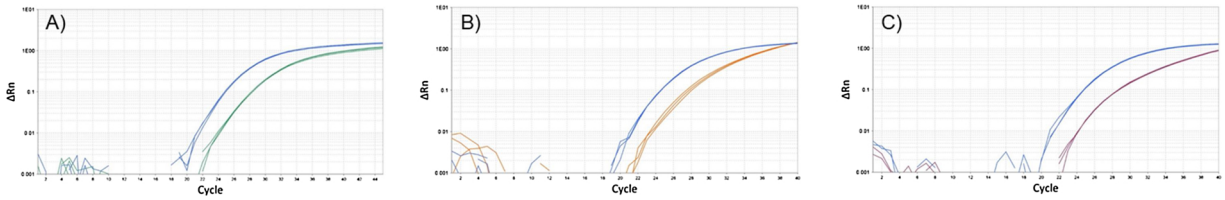

Fig. A3. There was a > 10-fold difference between the reference method (blue) and the (A) M460 V (ΔCq = 4.1, green), (B) A594 V (ΔCq = 4.6, orange), and (C) L595S (ΔCq = 5.4, purple) methods. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

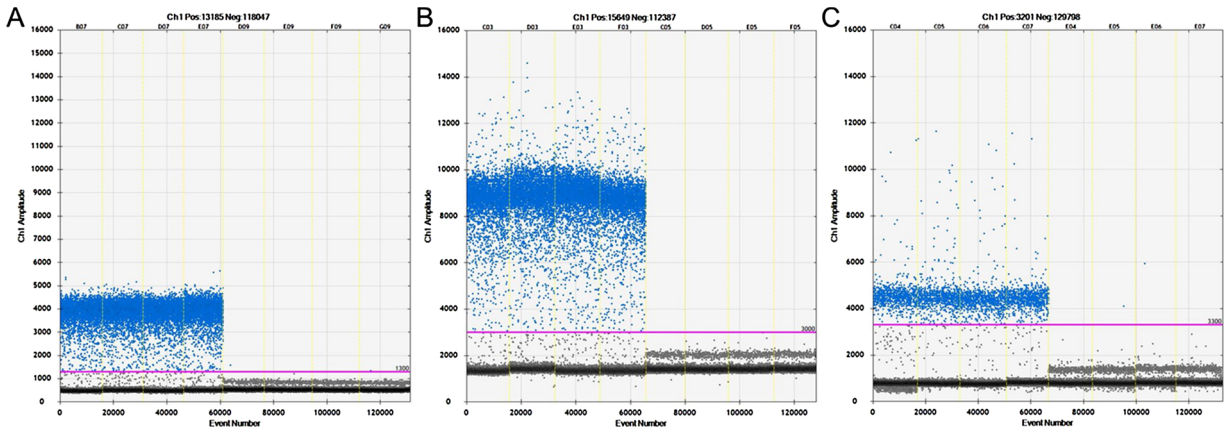

Fig. A4. A - method targeting the M460 V mutation; left-hand side sample containing the mutation, right-hand side sample containing the wild-type sequence. B - method targeting the A594 V mutation; left-hand side sample containing the mutation, right-hand side sample containing the wild-type sequence. C - method targeting the L595S mutation; left-hand side sample containing the mutation, right-hand side sample containing the wild-type sequence. All three methods exhibit cross-reactivity with the wild-type sequence, which is seen as the light grey cluster just above the negative cluster at the right-hand side of each panel.

Appendix B

Table B1
Information on the primers and probes.

Target	Type of oligonucleotide	5′-sequence-3’	Final concentration in PCR (nM)	Amplicon length (bp)
CMV (Sassenscheidt et al., 2006)	Forward primer	5′-GGCCTCGTAGTGAAAATTAATGGT-3’	600	72
	Reverse primer	5′-GGCCGTTACTGTCTGCAGGA-3’	600	
	Probe	5′-FAM-CCGTATTGGTGCGCGATCTGTTCAA-BHQ-3’	200	
A594V (adapted from Volfova et al., 2014)	Forward primer	5′-ACGGAGGCGTTGCTCTTTAA-3’	900	75
	Reverse primer	5′-GGAGCAGTGCGTGAGCTTG-3’	900	
	Probe	5′-FAM-CTCCAACACGCGGC-MGBNFQ-3’	300	
L595S (adapted from Volfova et al., 2014)	Forward primer	5′-ACGGAGGCGTTGCTCTTTAA-3’	900	75
	Reverse primer	5′-GGAGCAGTGCGTGAGCTTG-3’	900	
	Probe	5′-FAM-TTCTCCGACGCGCGG-MGBNFQ-3’	300	
M460V (this study)	Forward primer	5′-TGTTTCATCACGACCAGTGGAA-3’	900	163
	Reverse primer	5′-CTGGGGTTGTACGGGTTCAC-3’	900	
	Probe	5′-FAM-ACGTTCACGGGTGTAA-MGBNFQ-3’	300	

Table B2
dMIQE checklist.

Item	Importance	Included	Comments
Experimental design			
Definition of experimental and control groups	E [a]	Yes	
Number within each group	E	Yes	
Assay carried out by core laboratory or investigator's laboratory?	D [b]	Yes	Investigator’s laboratory
Power analysis	D	Yes	
Sample			
Description	E	Yes	
Volume or mass of sample processed	E	Yes	
Microdissection or macrodissection	E	No	
Processing procedure	E	No	
If frozen—how and how quickly?	E	No	
If fixed—with what, how quickly?	E	No	
Sample storage conditions and duration (especially for formalin-fixed, paraffin-embedded samples)	E	Yes	All samples stored at −20 ˚C
Nucleic acid extraction			
Procedure and/or instrumentation	E	Yes	
Storage conditions: temperature, concentration, duration, buffer	E	Yes	All nucleic acids stored at −20 ˚C, stock synthetic DNAs reconstituted in TE buffer
DNA or RNA quantification	E	No	
Quality/integrity, instrument/method, e.g. RNA integrity/R quality index and trace or 3′:5′	E	No	
Template structural information	E	Yes	Appendix B – Table B1
Template modification (digestion, sonication, preamplification, etc.)	E	No	
Template treatment (initial heating or chemical denaturation)	E	No	
DNA contamination assessment of RNA sample	E	No	
Inhibition dilution or spike	E	No	
Details of DNase treatment where performed	E	No	
Manufacturer of reagents used and catalogue number	D	Yes	High Pure viral nucleic acid kit (Roche)11858874001
Storage of nucleic acid: temperature, concentration, duration, buffer	E	Yes	
RT (If necessary)			
cDNA priming method + concentration	E	No	
One- or 2-step protocol	E	No	
Amount of RNA used per reaction	E	No	
Detailed reaction components and conditions	E	No	
RT efficiency	D	No	
Estimated copies measured with and without addition of RT	D	No	
Manufacturer of reagents used and catalogue number	D	No	
Reaction volume (for 2-step RT reaction)	D	No	
Storage of cDNA: temperature, concentration, duration, buffer	D	No	
dPCR target information			
Sequence accession number	E	Yes	For the design of synthetic DNAs the following sequence was used AY315197.
Amplicon location	D	Yes	Appendix C - Data C1-C3
Amplicon length	E	Yes	Appendix B - Table B1

(*continued on next page*)

Table B2 (*continued*)

Item	Importance	Included	Comments
In silico specificity screen (BLAST, others)	E	Yes	
Pseudogenes, retropseudogenes or other homologues?	D	No	
Sequence alignment	D	No	
Secondary structure analysis of amplicon and GC content	D	No	
Location of each primer by exon or intron (if applicable)	E	No	
Where appropriate, which splice variants are targeted?	E	No	
dPCR oligonucleotides			
Primer sequences and/or amplicon context sequence	E	Yes	Appendix B - Table B1
RTPrimerDB (real-time PCR primer and probe database) identification number	D	No	
Probe sequences	D	Yes	Appendix B - Table B1
Location and identity of any modifications	E	Yes	Fluorophore was changed from VIC to FAM, the same reverse primer was used for both A594 V and L595S assays
Manufacturer of oligonucleotides	D	Yes	Eurofins MWG Operon and Applied Biosystems
Purification method	D	Yes	Desalting (HPSF®)
dPCR protocol			
Complete reaction conditions	E	Yes	
Reaction volume and amount of RNA/cDNA/DNA	E	Yes	
Primer, (probe), Mg^{2+} and dNTP concentrations	E	Yes	Applicable for primers and probes, other chemicals were part of ddPCR™ Supermix for Probes (No dUTP) (concentrations not disclosed by the manufacturer)
Polymerase identity and concentration	E	No	Not disclosed by the manufacturer
Buffer/kit catalogue no. and manufacturer	E	Yes	#186 − 3024, Bio-Rad (Hercules, CA, USA)
Exact chemical constitution of the buffer	D	No	Not disclosed by the manufacturer
Additives (SYBR green I, DMSO, others)	E	No	Not disclosed by the manufacturer
Plates/ tubes Catalogue No and manufacturer	D	Yes	#0030128605, Eppendorf (Hamburg, Germany)
Complete thermocycling parameters	E	Yes	
Reaction setup	D	Yes	
Gravimetric or volumetric dilutions (manual/ robotic)	D	Yes	Gravimetric and volumetric manual dilutions
Total PCR reaction volume prepared	D	Yes	10 % larger volume was prepared
Partition number	E	Yes	Data available upon request
Individual partition volume	E	Yes	0.85 nL
Total volume of the partitions measured (effective reaction size)	E	Yes	Data available upon request
Partition volume variance/SD	D	No	
Comprehensive details and appropriate use of controls	E	Yes	
Manufacturer of dPCR instrument	E	Yes	
dPCR validation			
Optimisation data for the assay	D	No	
Specificity (when measuring rare mutations, pathogen sequences)	E	Yes	*In-silico* specificity was checked, *in vitro* specificity was partially assessed by checking the cross-reactivity od the mutation to the wild-type and on two EQA samples (wild-type CMV in plasma) but need to be further evaluated on a larger sample size.
Limit of detection of calibration control	D	No	
If multiplexing, comparison with singleplex assays	E	No	Multiplexing was not performed.
Data Analysis			
Mean copies per partition (λ or equivalent)	E	Yes	Data available upon request
dPCR analysis programme (source, version)	E	Yes	QuantaSoft v. 1.7.4.0917 (Bio-Rad; Hercules, CA, USA)
Outlier identification and disposition	E	Yes	Appendix B – TableB4
Results of no-template controls	E	Yes	All results of no-template controls were negative.
Examples of positive(s) and negative experimental results as supplemental data	E	Yes	Appendix A - Fig. A3
Where appropriate, justification of number and choice of reference genes	E	No	
Where appropriate, description of normalisation method	E	No	
Number and concordance of biological replicates	D	No	
Number and stage (RT or dPCR) of technical replicates	E	Yes	Materials and methods
Repeatability (intra-assay variation)	E	Yes	Results and discussion
Reproducibility (interassay/ user/ laboratory variation)	D	Yes	Results and discussion
Experimental variance or CI	E	Yes	Data available upon request
Statistical methods used for analysis	E	Yes	No specific statistical methods were needed (data were analysed by Poisson statistics in Microsoft Excel spreadsheet)
Data submission using RDML (real-time PCR data mark-up language)	D	No	

[a] essential; [b] desirable.

Table B3
Synthetic DNA characterisation in terms of copies per µL (cp/µL) by reference method targeting *UL54*.

Synthetic DNA	Stock (cp/µL)	Dilution 1 (cp/µL)	Dilution 2 (cp/µL)
DNA1	4.6E + 09	3.5E + 07	335,000
DNA2	4.4E + 09	3.4E + 07	292,000
DNA3	5.5E + 09	4.1E + 07	390,000

Table B4
Results of all three methods as copies per reaction (cp/rnx), with outliers highlighted in bold.

Dilution	M460V(cp/rnx)			A594V(cp/rnx)			L595S(cp/rnx)		
	Experiment #1	Experiment #2	Experiment #3	Experiment #1	Experiment #2	Experiment #3	Experiment #1	Experiment #2	Experiment #3
1	6398	6839	5979	7498	7197	6756	10,848	16,031	11,732
	6530	6683	6452	7143	6600	6537	17,174	15,962	17,743
	6469	**2213**	6266	7341	7219	6863	17,360	16,793	15,794
	6527	6479	6106	7296	6780	6785	15,535	15,165	19,007
	6242	6365	6206	7358	7031	7124	15,578	15,268	18,378
2	2854	2815	2794	3183	2837	2776	1217	1101	1203
	2869	2708	2621	3227	2814	2994	1194	1056	1310
	2713	2692	2730	3153	3089	3023	1310	1231	1271
	2853	2796	2771	3180	2981	3012	1203	1032	999
	2620	2771	2673	2985	3016	3158	1331	1157	1125
3	1149	830	874	1275	907	894	369	370	312
	1197	865	917	1395	876	922	337	373	422
	1142	803	921	1231	894	990	339	421	390
	1218	852	849	1286	892	878	312	411	**187**
	1171	871	931	1305	891	882	268	408	484
4	**114**	60	82	88	67	90	33	40	24
	75	75	76	102	82	91	41	46	32
	83	78	**126**	107	62	66	30	40	48
	100	74	77	**147**	65	81	37	40	40
	87	62	62	103	68	78	36	32	37
5	47	28	46	28	23	32	22	21	9
	63	20	47	47	28	42	22	21	11
	40	28	40	42	19	36	30	19	13
	36	33	40	38	23	39	9	23	13
	28	24	38	32	28	35	11	13	11
6	29	31	25	22	23	14	6	8	6
	30	17	13	21	18	18	7	**20**	13
	19	16	18	23	15	17	Neg. [a]	9	4
	15	12	9	23	14	24	12	10	Neg. [a]
	24	24	15	19	13	16	13	6	7
7	8	Neg. [a]	4	4	6	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]
	9	Neg. [a]	4	6	Neg. [a]	6	Neg. [a]	Neg. [a]	Neg. [a]
	4	Neg. [a]	4	7	4	Neg. [a]	Neg. [a]	Neg. [a]	4
	9	6	5	Neg. [a]	4	4	Neg. [a]	Neg. [a]	Neg. [a]
	7	Neg. [a]	Neg. [a]	7	Neg. [a]	Neg. [a]	Neg. [a]	4	Neg. [a]
8	Neg. [a]	4	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]
	4	Neg. [a]	Neg. [a]	4	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]
	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]
	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]
	4	4	Neg. [a]	Neg. [a]	4	Neg. [a]	Neg. [a]	Neg. [a]	Neg. [a]

[a] Neg., Reaction with less than three positive partitions.

Table B5
Limits of detection (green highlight) and quantification (blue highlight).

Mutation	Dilution	Assigned value (cp/rnx)	Experiment 1 (cp/rnx)	Experiment 1 CV (%)	Experiment 2 (cp/rnx)	Experiment 2 CV (%)	Experiment 3 (cp/rnx)	Experiment 3 CV (%)	Mean (cp/rnx)	CV (%) all
M460V	1	5600	6433	1.86	6591	3.20	6202	2.85	6396	3.5
	2	2800	2782	3.96	2756	1.97	2718	2.62	2752	2.9
	3	933	1176	2.74	844	3.30	898	3.90	973	15.7
	4	93	86	11.88	70	11.47	75	11.64	76	14.3
	5	47	43	30.71	27	19.00	42	9.83	37	29.6
	6	23	23	27.36	20	36.71	16	38.69	20	35.1
	7	8	7	24.85	2[a]	ND	4[a]	ND	6[a]	ND
	8	3	3[a]	ND	3[a]	ND	ND	ND	4[a]	ND
A594V	1	5600	7327	1.74	6965	3.87	6813	3.11	7035	4.2
	2	2800	3146	2.98	2947	4.01	2993	4.60	3029	4.6
	3	933	1298	4.65	892	1.23	913	5.06	1034	19.1
	4	93	100	8.03	69	11.11	81	12.45	82	18.5
	5	47	37	19.79	24	15.60	37	11.03	33	24.4
	6	23	22	7.28	17	23.20	18	22.76	19	20.1
	7	8	5[a]	ND	3[a]	ND	3[a]	ND	5[a]	ND
	8	3	2[a]	ND	1[a]	ND	2[a]	ND	4[a]	ND
L595S	1	15300	15299	17.2	15844	4.2	16531	17.8	15891	13.9
	2	1530	1251	5.1	1116	7.2	1181	10.5	1183	8.7
	3	510	325	11.6	397	5.9	402	17.8	373	15.1
	4	51	36	11.3	40	13.0	36	24.0	37	16.4
	5	26	19	45.3	20	20.4	12	13.5	17	37.8
	6	13	8[a]	ND	11	52.0	6[a]	69.0	8[a]	ND
	7	3	ND	ND	2[a]	ND	4[a]	ND	1[a]	ND
	8	1	ND	ND	ND	ND	ND	ND	ND.	ND

[a] At least one replicate was negative. ND, not determined due to negative replicate(s).

[a] At least one replicate was negative. ND, not determined due to negative replicate(s).

Table B6
Bias of the experimentally determined copy numbers relative to the assigned values.

Mutation	Dilution	Assigned value (cp/rnx)	Mean (cp/rnx)	Bias to assigned (%)
M460V	1	5600	6396	14.21
	2	2800	2752	−1.71
	3	933	973	4.25
	4	93	76	−18.17
	5	47	37	−20.71
	6	23	20	−14.29
	7	8	< LOD	ND
	8	3	< LOD	ND
A594V	1	5600	7035	25.63
	2	2800	3029	8.18
	3	933	1034	10.79
	4	93	82	−12.03
	5	47	33	−29.29
	6	23	19	−18.57
	7	8	< LOD	ND
	8	3	< LOD	ND
L595S	1	15,300	15,891	3.86
	2	1530	1183	−22.68
	3	510	373	−26.86
	4	51	37	−27.45
	5	26	17	−33.33
	6	13	< LOD	ND
	7	3	< LOD	ND
	8	1	Neg.	ND

< LOD, below the limit of detection, at least one replicate was negative; ND, not determined due to negative replicate(s); Neg., all replicates were negative.

Table B7
Results of INSTAND external quality assessment scheme 349 term 2016: 1 qPCR pre-run (vial 1, extraction 1).

Sample	Target	Technical repeat	Ct
349005	*UL54*	1	28.176
	(reference method)	2	28.251
	M460V	1	33.439
		2	33.437
	A594V	1	Undetermined
		2	Undetermined

(*continued on next page*)

Table B7 (*continued*)

Sample	Target	Technical repeat	Ct
349006	*UL54*	1	28.704
	(reference method)	2	29.102
	M460V	1	33.658
		2	33.148
	A594V	1	Undetermined
		2	Undetermined
349007	*UL54*	1	28.691
	(reference method)	2	28.522
	M460V	1	Undetermined
		2	Undetermined
	A594V	1	30.824
		2	30.863
349008	*UL54*	1	28.824
	(reference method)	2	28.532
	M460V	1	Undetermined
		2	Undetermined
	A594V	1	Undetermined
		2	Undetermined

Table B8
Results of the dPCR of the INSTAND EQA scheme 349 terms 2016, 2017 and 2018.

Study	Matrix	Sample	*UL54* (reference method)		M460V		A594V		L595S	
			Mean (cp/mL plasma)	CV (%)	Mean (cp/mL plasma)	CV (%)	Mean (cp/mL plasma)	CV (%)	Mean (cp/mL plasma)	CV (%)
2016	Plasma	349005	27,694	8.03	7524	17.47	neg	ND	NA	NA
		349006	23,915	15.4	8245	31.16	neg	ND	NA	NA
		349007	28,245	15.0	neg	ND	19,013	17.52	NA	NA
		349008	*dPCR not preformed*							
2017	Plasma	349009	43,345	39.82	neg	ND	18,345	40.5	NA	NA
		349010	46,417	14.01	neg	ND	neg	ND	NA	NA
		349011	53,142	21.41	neg	ND	22,113	22.52	NA	NA
		349012	49,483	6.83	10,790	7.67	neg	ND	NA	NA
2018	Plasma	349016	127,472	3.92	NA	NA	NA	NA	111,810	4.84

Neg., all replicates were negative, ND, not determined due to negative replicates, * samples 349009 and 349,011 are identical, NA, not applicable as the mutaitons was not a part of the scheme.

Table B9
Fractional abundance of the mutation (mutation %) in regards to the overall virus for the INSTAND EQA samples term 2016.

Sample	Vial	Extraction	Mutation		*UL54* (reference method)	Mutation %	Mean mutation %	Mutation % CV (%)
			Identification	(cp/mL plasma)	(cp/mL plasma)			
349005	1	1	M460V	7437	29,381	25.31	27.16	12.70
		2		9201	29,194	31.52		
	2	1		6938	24,637	28.16		
		2		6519	27,563	23.65		
349006	1	1	M460V	[a]	21,980	[a]	33.53	27.12
		2		5856	25,238	23.20		
	2	1		7468	20,152	37.06		
		2		11,412	28,291	40.34		
349007	1	1	A594V	16,453	26,153	62.91	67.18	6.61
		2		24,303	34,857	69.72		
	2	1		18,105	25,107	72.11		
		2		17,190	26,863	63.99		

ND not determined due to negative reactions, [a] mistake in reaction preparation.

Table B10
Fractional abundance of the mutation (mutation %) in regards to the overall virus for the INSTAND EQA samples term 2017.

Sample	Vial	Extraction	Mutation		*UL54* (reference method)	Mutation %	Mean mutation %	Mutation % CV (%)
			Identification	(cp/mL plasma)	(cp/mL plasma)			
349009 = 349011	1	1	A594V	14,520	35,598	40.79	41.56	2.46
		2		25,939	63,625	40.77		
	2	1		23,478	56,245	41.74		
		2		24,516	57,100	42.93		
349010	1	1	Neg.	Neg.	45,617	ND	ND	ND
		2		Neg.	52,034	ND		
	2	1		Neg.	38,954	ND		
		2		Neg.	49,062	ND		
349011 = 349009	1	1	A594V	7240	16,993	42.61	42.34	2.33
		2		25,787	60,436	42.67		
	2	1		20,889	48,375	43.18		
		2		19,465	47,576	40.91		
349012	1	1	M460V	10,462	45,928	22.78	21.87	7.93
		2		[a]	[a]	ND		
	2	1		10,526	52,966	19.87		
		2		11,383	49,555	22.97		

ND not determined due to negative reactions, [a] mistake in reaction preparation, Neg. negative reaction/sample.

Table B11
Fractional abundance of the mutation (mutation %) in regards to the overall virus for the INSTAND EQA samples term 2018.

Sample	Vial	Extraction	Mutation		*UL54* (reference method)	Mutation %	Mean mutation %	Mutation % CV (%)
			Identification	(cp/mL plasma)	(cp/mL plasma)			
349016	1	1	L595S	117,417	130,594	89.91	87.71	3.45
		2		114,045	130,963	87.08		
	2	1		106,606	128,575	82.91		
		2		112,000	123,746	90.51		

Appendix C

Data C1. FASTA sequences of synthetic DNA1, containing part of the *UL54* region (grey) with amplified fragment (red), and part of the *UL97* region (white) with the M460 V mutation (triplet marked in green) and A594 V mutation (triplet marked in magenta).

AGCGAGACGGTGCTCACGGTCTGGATGTCGGGCCTGATCCGCACGCGCGCCGCTGGCGAGCAACAGCAGC
CGCCGTCGCTGGTGGGTACGGGCGTGCACCGCGGTCTGCTCACGGCCACGGGCTGCTGTCTGCTGCACAA
CGTCACGGTACATCGACGTTTCCACACAGACATGTTTCATCACGACCAGTGGAAGCTGGCGTGCATCGAC
AGCTACCGACGTGCCTTTTGCACGTTGGCCGACGCTATCAAATTTCTCAATCACCAGTGTCGTGTATGCC
ACTTTGACATTACACCCGTGAACGTGCTCATCGACGTGAACCCGCACAACCCCAGCGAGATCGTGCGCGC
CGCGCTGTGCGATTACAGCCTCAGCGAGCCCTATCCGGATTACAACGAGCGCTGTGTGGCCGTCTTTCAG
GAGACGGGCACGGCGCGCCGCATCCCCAACTGCTCGCACCGTCTGCGCGAATGTTACCACCCTGCTTTCC
GACCCATGCCGCTGCAGAAGCTGCTCATCTGCGACCCGCACGCGCGTTTCCCCGTAGCCGGTCTACGGCG
TTATTGCATGTCGGAGCTGTCGGCGCTGGGCAACGTGCTGGGCTTTTGCCTCATGCGGCTGTTGGACCGG
CGCGGTCTGGACGAGGTGCGCATGGGCACGGAGGCGTTGCTCTTTAAGCACGCCGGCGCGGCCTGCCGCG
TGTTGGAGAACGGCAAGCTCACGCACTGCTCCGACGCCTGTCTGCTCATTCTGGCGGCGCAAATGAGCTA
CGGCGCCTGTCTCCTGGGCGAGCATGGCGCCGCGCTGGTGTCGCACACGCTGCGCTTTGTGGAGGCCAAG
ATGTCCTCGTGTCGCGTACGCGCCTTTCGCCGCTTCTACCACGAATGCTCGCAGACCATGCTGCACGAAT
ACGTCAGAAAGAACGTGGAGCGTCTGTTGGCCACGAGCGACGGGCTGTATTTATATAACGCCTTTCGGCG
CACCACCAGCATAATCTGCGAGGAGGACCTTGACGGTGACTGCCGCCAACTGTTCCCCGAGTAATCAAAG
ATGACACGCCGCAACGGAATTTTAGCCAGCCGCGCGATGGCCCCGGCCTCGTAGTGAAAATTAATGGTGT
TGAACAGATCGCGCACCAATACGGCGTCCTGCAGACAGTAACGGCCTACCTGGGCGCGGCCCTCGGCATT
AGCCACGAAACAACGCGGGATGTCCT

Data C2. FASTA sequences of synthetic DNA2, containing part of the *UL54* region (grey) with amplified fragment (red), and part of the *UL97* region (white) with L595S mutation (triplet marked in green).

AGCGAGACGGTGCTCACGGTCTGGATGTCGGGCCTGATCCGCACGCGCGCCGCTGGCGAGCAACAGCAGC
CGCCGTCGCTGGTGGGTACGGGCGTGCACCGCGGTCTGCTCACGGCCACGGGCTGCTGTCTGCTGCACAA
CGTCACGGTACATCGACGTTTCCACACAGACATGTTTCATCACGACCAGTGGAAGCTGGCGTGCATCGAC
AGCTACCGACGTGCCTTTTGCACGTTGGCCGACGCTATCAAATTTCTCAATCACCAGTGTCGTGTATGCC
ACTTTGACATTACACCCATGAACGTGCTCATCGACGTGAACCCGCACAACCCCAGCGAGATCGTGCGCGC
CGCGCTGTGCGATTACAGCCTCAGCGAGCCCTATCCGGATTACAACGAGCGCTGTGTGGCCGTCTTTCAG
GAGACGGGCACGGCGCGCCGCATCCCCAACTGCTCGCACCGTCTGCGCGAATGTTACCACCCTGCTTTCC
GACCCATGCCGCTGCAGAAGCTGCTCATCTGCGACCCGCACGCGCGTTTCCCCGTAGCCGGTCTACGGCG
TTATTGCATGTCGGAGCTGTCGGCGCTGGGCAACGTGCTGGGCTTTTGCCTCATGCGGCTGTTGGACCGG
CGCGGTCTGGACGAGGTGCGCATGGGCACGGAGGCGTTGCTCTTTAAGCACGCCGGCGCGGCCTGCCGCG
CGTCGGAGAACGGCAAGCTCACGCACTGCTCCGACGCCTGTCTGCTCATTCTGGCGGCGCAAATGAGCTA
CGGCGCCTGTCTCCTGGGCGAGCATGGCGCCGCGCTGGTGTCGCACACGCTGCGCTTTGTGGAGGCCAAG
ATGTCCTCGTGTCGCGTACGCGCCTTTCGCCGCTTCTACCACGAATGCTCGCAGACCATGCTGCACGAAT
ACGTCAGAAAGAACGTGGAGCGTCTGTTGGCCACGAGCGACGGGCTGTATTTATATAACGCCTTTCGGCG
CACCACCAGCATAATCTGCGAGGAGGACCTTGACGGTGACTGCCGCCAACTGTTCCCCGAGTAATCAAAG
ATGACACGCCGCAACGGAATTTTAGCCAGCCGCGCGATGGCCCCGGCCTCGTAGTGAAAATTAATGGTGT
TGAACAGATCGCGCACCAATACGGCGTCCTGCAGACAGTAACGGCCTACCTGGGCGCGGCCCTCGGCATT
AGCCACGAAACAACGCGGGATGTCCT

Data C3. FASTA sequences of synthetic DNA3, containing part of the *UL54* region (grey) with amplified fragment (red), and part of the wild-type *UL97* region (white).

AGCGAGACGGTGCTCACGGTCTGGATGTCGGGCCTGATCCGCACGCGCGCCGCTGGCGAGCAACAGCAGC
CGCCGTCGCTGGTGGGTACGGGCGTGCACCGCGGTCTGCTCACGGCCACGGGCTGCTGTCTGCTGCACAA
CGTCACGGTACATCGACGTTTCCACACAGACATGTTTCATCACGACCAGTGGAAGCTGGCGTGCATCGAC
AGCTACCGACGTGCCTTTTGCACGTTGGCCGACGCTATCAAATTTCTCAATCACCAGTGTCGTGTATGCC
ACTTTGACATTACACCCATGAACGTGCTCATCGACGTGAACCCGCACAACCCCAGCGAGATCGTGCGCGC
CGCGCTGTGCGATTACAGCCTCAGCGAGCCCTATCCGGATTACAACGAGCGCTGTGTGGCCGTCTTTCAG
GAGACGGGCACGGCGCGCCGCATCCCCAACTGCTCGCACCGTCTGCGCGAATGTTACCACCCTGCTTTCC
GACCCATGCCGCTGCAGAAGCTGCTCATCTGCGACCCGCACGCGCGTTTCCCCGTAGCCGGTCTACGGCG
TTATTGCATGTCGGAGCTGTCGGCGCTGGGCAACGTGCTGGGCTTTTGCCTCATGCGGCTGTTGGACCGG
CGCGGTCTGGACGAGGTGCGCATGGGCACGGAGGCGTTGCTCTTTAAGCACGCCGGCGCGGCCTGCCGCG
CGTTGGAGAACGGCAAGCTCACGCACTGCTCCGACGCCTGTCTGCTCATTCTGGCGGCGCAAATGAGCTA
CGGCGCCTGTCTCCTGGGCGAGCATGGCGCCGCGCTGGTGTCGCACACGCTGCGCTTTGTGGAGGCCAAG
ATGTCCTCGTGTCGCGTACGCGCCTTTCGCCGCTTCTACCACGAATGCTCGCAGACCATGCTGCACGAAT
ACGTCAGAAAGAACGTGGAGCGTCTGTTGGCCACGAGCGACGGGCTGTATTTATATAACGCCTTTCGGCG
CACCACCAGCATAATCTGCGAGGAGGACCTTGACGGTGACTGCCGCCAACTGTTCCCCGAGTAATCAAAG
ATGACACGCCGCAACGGAATTTTAGCCAGCCGCGCGATGGCCCCGGCCTCGTAGTGAAAATTAATGGTGT
TGAACAGATCGCGCACCAATACGGCGTCCTGCAGACAGTAACGGCCTACCTGGGCGCGGCCCTCGGCATT
AGCCACGAAACAACGCGGGATGTCCT

Data C4. Specificity assessment. Primer-BLAST search for unintended amplicons yielded two results for M460 V.

```
product length = 163
Forward primer  1       TGTTTCATCACGACCAGTGGAA  22
Template        143812  .C..C..C........C.....  143833

Reverse primer  1       CTGGGGTTGTACGGGTTCAC  20
Template        143974  .........CG.........  143955

>AF403738.1 Culex nigripalpus baculovirus, complete genome


product length = 292
Forward primer  1      TGTTTCATCACGACCAGTGGAA  22
Template        33774  .C.....A.T.A..........  33753

Reverse primer  1      CTGGGGTTGTACGGGTTCAC  20
Template        33483  A.TT.....A..........  33502
```

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