Cover page

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Running title: A Precision Approach to HCMV Drug Resistance

i. Chapter Title

Digital PCR-based Genotyping: A Precision Approach to HCMV Drug Resistance

ii. Summary/Abstract

The genotyping workflow described uses digital PCR (dPCR) to detect and quantify drug resistance mutations in human cytomegalovirus (HCMV). The method focuses on the detection and quantification of three common mutations in the UL97 gene at codons 460, 594 and 595, which are responsible for the majority of ganciclovir-resistant clinical isolates. The dPCR approach offers high sensitivity and accuracy, making it suitable for routine testing as well as a reference measurement procedure for external quality assessment schemes. The workflow includes several key steps: DNA isolation, preparation of the dPCR reaction mixture, partitioning, thermocycling and data analysis. This method improves the detection capabilities of HCMV drug resistance and provides a robust and efficient tool for clinical and research applications.

iii. Key Words.

digital PCR, human cytomegalovirus, antimicrobial drug-resistance, mutations

1. Introduction

Human cytomegalovirus (HCMV), or human herpesvirus 5 (HHV-5), is a member of the *Betaherpesvirinae* subfamily and, like other herpesviruses, can establish lifelong latent infections in humans. In healthy, immunocompetent individuals, HCMV is controlled by a vigorous immune response resulting in an asymptomatic infection or causing mild symptoms, while 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 [1–3]. Despite enormous efforts to develop vaccines, there is no licensed HCMV vaccine, yet. The gold standard treatment for preventing HCMV diseases is the antiviral drug ganciclovir. However, a prolonged antiviral therapy, suboptimal drug levels, high levels of immunosuppression and lack of HCMV immunity, can cause development of antiviral resistance due to mutations in UL97 gene [4–6]. UL97 gene codes for the viral protein kinase, which phosphorylates ganciclovir into its active form of

ganciclovir monophosphate, thus mutations in this gene prevent phosphorylation of the pro-drug, providing drug resistance to viral strains.

For routine genotyping, Sanger sequencing is used for detection of viral mutations that confer drug resistance and it is expected that next generation sequencing will prevail due to its higher capacity [5]. Another approach, genotyping by dPCR, can provide a rapid, sensitive and accurate method for detection and quantification of the most common resistance-conferring mutations on one hand, and support the standardisation of next generation sequencing approaches for genotyping on the other. Here we describe three dPCR methods used for detection and quantification of common mutations at codons 460, 594 and 595 of UL97, which account for 70 % of ganciclovir-resistant clinical isolates [7, 8], as well as the reference measurement procedure for HCMV quantification [9, 10]. The analytical method described below is intended for the accurate quantification of control or reference materials or external quality assessment schemes. For this purpose, two or more vials of one batch of the sample are used. If the method is to be used for routine tests, the number of vials, technical parallels can be reduced, and sample dilution step omitted.

2. Materials

2.1 DNA isolation

1. Biosafety cabinet level 2
2. Water bath/heating block
3. Vortex
4. Centrifuge, max speed at least 13000 g
5. Pipettes with corresponding tips
6. High Pure Viral Nucleic Acid Kit (Roche) (see **Note 1**)
7. Absolute ethanol (see **Note 2**)
8. Nuclease-free water (see **Note 3**)
9. Nuclease-free 1.5 mL microcentrifuge tube
10. DNA low bind 1.5 mL microcentrifuge tubes (see **Note 4**)

2.2 dPCR reaction set up

1. UV chamber
2. Pipettes with corresponding tips
3. Vortex and microcentrifuge
4. ddPCR™ Supermix for Probes (No dUTP) (Bio-Rad)
5. Nuclease-free water
6. Oligonucleotides (Table 1)

2.3 Generation of partitions and thermal cycling

1. UV chamber
2. Pipettes (Rainin) with corresponding tips (*see* **Note 5**)
3. Holders for cartridges (Bio-Rad)
4. DG8 cartridges (Bio-Rad)
5. DG8 gaskets (Bio-Rad)
6. Droplet generator oil (Bio-Rad)
7. 96-well semi-skirted microtiter plate (e.g. Eppendorf)
8. Cooling block
9. Pierceable Foil Heat Seal (Bio-Rad)
10. Heat Sealer (e.g. Bio-Rad PX1 PCR Plate Sealer)
11. Thermal cycler (e.g. C1000 or T100 Touch Thermal, Bio-Rad)

2.4 Partition read out and analysis

1. Droplet reader oil (Bio-Rad)
2. QX200 Droplet Reader (Bio-Rad)
3. QuantaSoft software (Bio-Rad)
4. Excel (Microsoft) or similar software

3. Methods

3.1 DNA isolation

1. Work in the biosafety cabinet in a biosafety level 2 laboratory.
2. Extract each sample in 2 parallels. Use 200 µL of undiluted primary sample (cell culture lysate, whole blood, serum, plasma) for each isolation.
3. Included one negative control of isolation (NCI) in each series to demonstrate the absence of sample or kit contamination during isolation (**Note 6**).
4. Follow the isolation protocol High Pure Viral Nucleic Acid Kit manual [11] (see **Notes 1-3**).
5. Use isolated DNA directly or store it at -20 °C.

3.2 Analysis setup

1. QX200 platform enables a maximum of 96 reactions in one rune (96 well plate). An example of a plate setup in Excel for analysis of two samples (A and B), two vials each is shown in Figure 1.
2. Calculate the volume of PCR reactions needed for analysis of all samples. Each final PCR reaction should consist of 10 µL of ddPCR Supermix for probes (No dUTP), 6 µL of primers and probes in appropriate working concentration to achieve final concentrations as described in Table 1 (see **Notes 7** and **8**) and 4 µL of DNA. Due to the loss of volume in the pipetting steps 20 % surplus should be prepared, when calculating volumes. Consequently, if e.g. 10 reactions are needed for analysis, the quantity of reagents needed should be calculated for 12 reactions (120 µL of ddPCR Supermix for probes (No dUTP) and 72 µL of PPS mix should be used, instead of 100 µL and 60 µL, respectively).

3.3 PCR reaction mixture preparation

1. The PCR reaction is prepared in a dedicated UV cabinet (see **Note 9**).

2. Thaw all reaction components; primers, probe or primer and probe mix and ddPCR Supermix for probes (No dUTP) (see **Note 10**) completely at room temperature and mix thoroughly before use. Exposure to light should be minimized.
3. Mix the adequate quantity of dedicated ddPCR Supermix and primers and probes, or primer and probe mixes (see **Note 11**) and transfer the mixes with PCR reaction mixtures to a UV cabinet dedicated for addition of nucleic acids.

Prepare an appropriate (e.g. 10-time) dilution of extracted DNA and add 4.4 µL of DNA, positive controls, NCI or no template controls (NTCs) to 17.6 µL of pre-reaction mixture to prepare a 10 % surplus of reaction mixture (**Note 12).** Mix and centrifuge.

3.4 Generation of partitions and thermal cycling

4. Turn on the droplet (partition) generator by connecting it to a power source.
5. Prepare holder(s) for cartridges, DG8 cartridges and DG8 gaskets.
6. Insert the cartridge into the cartridge holder.
7. Transfer 20 µL of the PCR reaction mixture to Sample wells (middle row) of the cartridge according to plan. Only 20 µl aerosol-barrier (filtered) Rainin pipet tips should be used in this step (see **Note 13**)
8. Transfer 70 µL of droplet generation oil into each Oil well (bottom row) of the cartridge.
9. Using the holes on the sides, hook the gasket over the cartridge holder.
10. Open QX200 Droplet Generator by pressing the button on the top of the to the instrument.
11. Place the holder with the cartridge into the instrument and check if cartridge status indicator light (middle) turned green.
12. Close the instrument by pressing the button on the top of the to the instrument. When the lid is closed, partition generation begins, indicated by flashing green light on the right. Partition generation is finished when all three indicator lights are solid green.
13. Open the instrument by pressing the button on the top and remove the holder with the cartridge.

14. Place the holder with the cartridge on a flat surface.
15. Remove and discard the gasket.
16. Transfer 40 µL of suspension with droplets (partitions) from Droplets well (top) into a 96-well plate placed on cooling block according to the plan (see **Note 14**).
17. Repeat droplet generation and transfer steps for all the samples.
18. After the transfer of all the samples to a 96-well plate seal the plate with pierceable foil using PCR plate sealer (see **Note 15**).
19. Place sealed 96-well plate in the PCR cycler. Run the program described in Table 2.

3.5 Partition read out and analysis

1. Turn on the droplet reader to warm up the instrument.
2. Start the QuantaSoft software on the connected computer.
3. Transfer the 96-well plate into the QX200 Droplet Reader.
4. Flush the instrument by selecting “Setup” -> “Instrument routines” -> “Flush System” in the QuantaSoft software.
5. To configure the plate layout select “Setup” -> “Template” -> “New”. Enter the new file name. Enter all the necessary information (sample names, targets, detection type, supermix) using well editor. Save the created template.
6. Click “Run” to initiate droplet reading. Set dye (FAM/HEX in our example) when the Run Options window appears.
7. After reading is finished, save the experiment data, and remove the plate from the instrument.
8. In the QuantaSoft software select “Setup” -> “Plate” -> “Load”. When the plate is loaded click “Analyze” to start data analysis.
9. In “1D Amplitude” view set the fluorescence thresholds for distinguishing between positive and negative droplets. The thresholds should be set simultaneously for all samples in a given assay. NTCs can be used as a guide for determining the negative population and threshold setting just above the negative cluster found in NTC(s) (see **Note 16**) (Figure 2).
10. Before further analysis quality control should be performed:

11. Wells with accepted number of partitions <10,000 should not be considered for further analysis.
12. The reaction in each well is considered as positive, if 3 or more partitions show the fluorescent signal above the threshold value.
13. NTC should be negative (see **Note 17**).
14. Positive control reaction should be positive (see **Note 17**).
15. NCI should be negative (see **Note 17**).
16. The software calculates the concentration of target copies in each sample based on the set thresholds. The results can be viewed under the "Concentration" tab and can be exported in .csv format for further analysis in spreadsheet software such as Excel (Microsoft).

4. Notes

1. According to the manufacturer's guidelines High Pure Viral Nucleic Acid Kit (Roche) should be stored at room temperature. Polymerase K and Poly(A) are lyophilized and can be stored at room temperature until they are dissolved in Elution Buffer. After reconstitution, both should be aliquoted and stored in dark and at -20 °C. The volume of the aliquots should be adapted to the expected number of parallel isolations (e.g. 550 µl of Polymerase K and 40 µl of Poly(A) for 10 parallel isolations).
2. Absolute ethanol is needed for the preparation of *Inhibitor Removal Buffer* and *Wash Buffer*.
3. Nuclease free water is used as a negative control of isolation (NCI).
4. DNA low bind (e.g. Eppendorf DNA LoBind tubes) microcentrifuge tubes are used to maximize recovery of DNA with minimal sample-to-surface binding.
5. According to recommendations from dPCR platform manufacturer, Rainin L-20 XLS 2-20 µL pipette and GP-L10F tips (Rainin 17002429; Tips LTS 20 µL Filter) are to be used for reaction mixture pipetting and Rainin L-50 XLS 5-50 µL pipette and GP-L200F tips (Rainin 17002428; Tips LTS 200 µL Filter) for droplet pipetting.
6. Negative controls of isolation comprise of the same volume of nuclease free water as is the volume of the sample e.g. if the sample volume is 200 µL then 200 µL of nuclease free water

will be needed as a NCI. NCI should be added in to each extraction as the last sample in the series. If 10 or more samples are in the extraction series, an additional NEC should be added e.g. for 12 samples, 2 NCI, for 24 samples 3, and so on.

7. If oligonucleotides are delivered lyophilized they need to be resuspended according to manufacturer's instruction to the concentration of 100 µM using nuclease free water. Lyophilized stock is initially shortly centrifuged to allow all of the content to sediment. Then it is resuspended with nuclease free water, well vortexed and centrifuged again. Exposure to light should be minimized. Oligonucleotides are stable up to 6 years when stored at -20 °C. Potential exception are probes with less stable fluorophores.
8. From stock solutions (100 µM) of oligonucleotides, working solutions of forward and reverse primers and probe can be prepared in a form of a primer and probe mix to decrease run to run variability and shorten the time for PCR set up. Below is an example of preparation of such mixes for oligonucleotides listed in Table 1 (calculations based on 6 µL mix added to final volume of 20 µL per reaction):
    - UL54: UL54 forward primer 36 µL, UL54 reverse primer 36 µL, UL54 probe 12 µL, nuclease free water 1716 µL
    - A594V: A594V forward primer 54 µL, A594V reverse primer, 54 µL, A594V probe 18 µL, nuclease free water 1674 µL
    - L595S: L595S forward primer 54 µL, L595S reverse primer, 54 µL, L595S probe 18 µL, nuclease free water 1674 µL
    - M460V: M460V forward primer 54 µL, M460V reverse primer, 54 µL, M460V probe 18 µL, nuclease free water 1674 µL

    Such mixes are stored at -20 °C and are stable for up to 6 years.
9. In order to prevent cross-contamination, it is recommended to perform preparation of PCR reaction mixture and addition of DNA at separate locations using dedicated laboratory equipment

10. ddPCR Supermix for probes (No dUTP) is a 2 times concentrated reaction mixture stable at -20 °C till the expiration date. Once thawed, it can be stored at 4 °C for up to two weeks.
11. For our example 240 µL of ddPCR Supermix and 132 µL of primers and probe mix should be prepared for each target.
12. To ensure that 20 µL of reaction mixture are transferred to the droplet generation process, the reaction mixture is prepared with a 10 % surplus. Diluted DNA should be used shortly after the preparation.
13. For best droplet generation air bubbles should be avoided and samples should wet the bottom of the wells, so they are wicked into microchannels. To achieve this, pipetting techniques described in the manufacturer's instruction manual (Catalog Number 1864002, Bio-Rad) should be followed. In short, pipette tip should be carefully slid along the wall at a 15 degree angle until it passing over the ridge near the bottom. Then the pipette tip should be drag against the bottom of the sample well while a small portion of the sample should be slowly dispensed. After dispensing about half of the sample, the tip should be slowly pull up the side wall while the rest of the sample should be dispensed.
14. To avoid evaporation, place the 96-well plate on a cooling block. To avoid shearing or coalescing the droplets (partitions) pipetting techniques described in the manufacturer's instruction manual (Catalog Number 1864002, Bio-Rad) should be followed. In short, an 8-channel manual L-50 pipet with 200 µL tips should be used. Pipet tips should be positioned at a 30-45° angle to the junction where the side wall meets the bottom of the Droplet wells. Suspension with the droplets (partitions) should be slowly drawn from the wells into the pipet tips. Pipette tips should be then positioned along the side of the wells of 96-well plate, near the bottom of the well, and suspension with the droplets should be slowly dispensed. Drawing and aspirating of droplets should take 5 s each. To prevent contamination full wells should be covered with 8-cap strips.
15. It is recommended to begin thermal cycling within 30 minutes of sealing the plate. Alternatively, sealed plates can be stored at 4 °C for up to 4 hours before thermal cycling.

16. In our setup, thresholds are 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 UL54 method at amplitude approximately 3000) in "1D Amplitude" view. As some cross reactivity was observed with wild type (see PC in Figure2), thresholds were set above the fog observed in PC.
17. The analysis is not valid, if the NTC(s) and/or NCI are positive or if the positive control reaction is negative. It is sufficient to test NCI only for the target that is expected in highest concentration (UL54 in our case).

5. References

1. Chevillotte M, von Einem J, Meier BM, et al (2010) A new tool linking human cytomegalovirus drug resistance mutations to resistance phenotypes. Antiviral Res 85:318–327. https://doi.org/10.1016/j.antiviral.2009.10.004
2. Seitz R (2010) Human Cytomegalovirus (HCMV) – Revised. Transfus Med Hemotherapy 37:365–375. https://doi.org/10.1159/000322141
3. Griffiths P, Reeves M (2021) Pathogenesis of human cytomegalovirus in the immunocompromised host. Nat Rev Microbiol 19:759–773. https://doi.org/10.1038/s41579-021-00582-z
4. Krishna BA, Wills MR, Sinclair JH (2019) Advances in the treatment of cytomegalovirus. Br Med Bull 131:5–17. https://doi.org/10.1093/bmb/ldz031
5. Chou S (2020) Advances in the genotypic diagnosis of cytomegalovirus antiviral drug resistance. Antiviral Res 176:104711. https://doi.org/10.1016/j.antiviral.2020.104711
6. Gilbert C, Boivin G (2005) Human cytomegalovirus resistance to antiviral drugs. Antimicrob Agents Chemother 49:873–883. https://doi.org/10.1128/AAC.49.3.873-883.2005
7. Boivin G, Gilbert C, Gaudreau A, et al (2001) Rate of Emergence of Cytomegalovirus (CMV) Mutations in Leukocytes of Patients with Acquired Immunodeficiency Syndrome Who Are Receiving Valganciclovir as Induction and Maintenance Therapy for CMV Retinitis. J Infect Dis 184:1598–1602. https://doi.org/10.1086/324672
8. Bogožalec Košir A, Cvelbar T, Kammel M, et al (2020) Digital PCR method for detection and quantification of specific antimicrobial drug-resistance mutations in human cytomegalovirus. J Virol Methods 281:113864. https://doi.org/10.1016/j.jviromet.2020.113864

9. Pavšič J, Devonshire A, Blejec A, et al (2017) Inter-laboratory assessment of different digital PCR platforms for quantification of human cytomegalovirus DNA. Anal Bioanal Chem 409:2601–2614. https://doi.org/10.1007/s00216-017-0206-0
10. Milavec M, Pavšič J, Bogožalec Košir A, et al (2021) The performance of human cytomegalovirus digital PCR reference measurement procedure in seven external quality assessment schemes over four years. Methods. https://doi.org/10.1016/j.ymeth.2021.03.016
11. Roche Diagnostics GmbH (2020) High Pure Viral Nucleic Acid Kit. Roche Diagnostics GmbH 20:3–11
12. Sassenscheidt J, Rohayem J, Illmer T, Bandt D (2006) Detection of β-herpesviruses in allogenic stem cell recipients by quantitative real-time PCR. J Virol Methods 138:40–48. https://doi.org/10.1016/j.jviromet.2006.07.015
13. Volfova P, Lengerova M, Lochmanova J, et al (2014) Detecting human cytomegalovirus drug resistant mutations and monitoring the emergence of resistant strains using real-time PCR. J Clin Virol 61:270–274. https://doi.org/10.1016/j.jcv.2014.07.008

Figure captions

Figure 1: An example of a plate setup for two EQA samples (A and B). Each sample is analysed in two vials (A1, A2, B1, B2), each vial in two extraction parallels (e.g. A1-1 and A1-2), each extraction parallel in 2 technical repeats prepared in Excel, (10x – 10 times diluted sample, PC – positive control, NCI – negative control of isolation, NTC - no template control).

Figure 2: An example of amplification plots: A- sample A tested with UL54 method; B - sample A tested with M460V mutation detection method; C – sample A tested with UL54 method; D - sample B tested with A594V mutation detection method (NCI – negative control of isolation, NTC – no template control, PC – positive control - synthetic DNA comprising all targeted sequences)

Table captions

Table 1: Primers and probes used in genotyping for antimicrobial drug resistance in HCMV

Table 2: PCR program for amplification of antimicrobial drug resistance and HCMV specific targets.

Figures

Figure 1

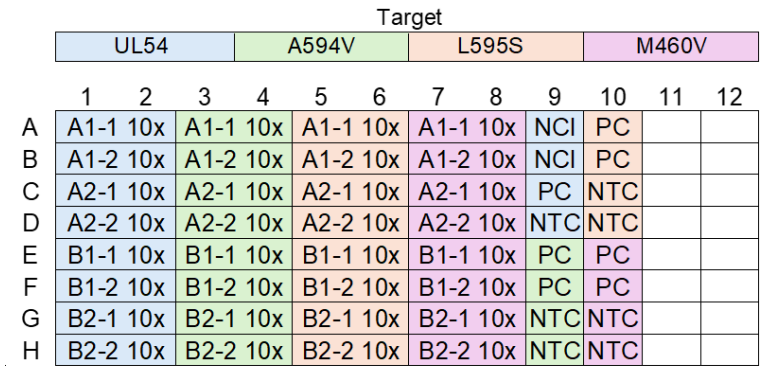
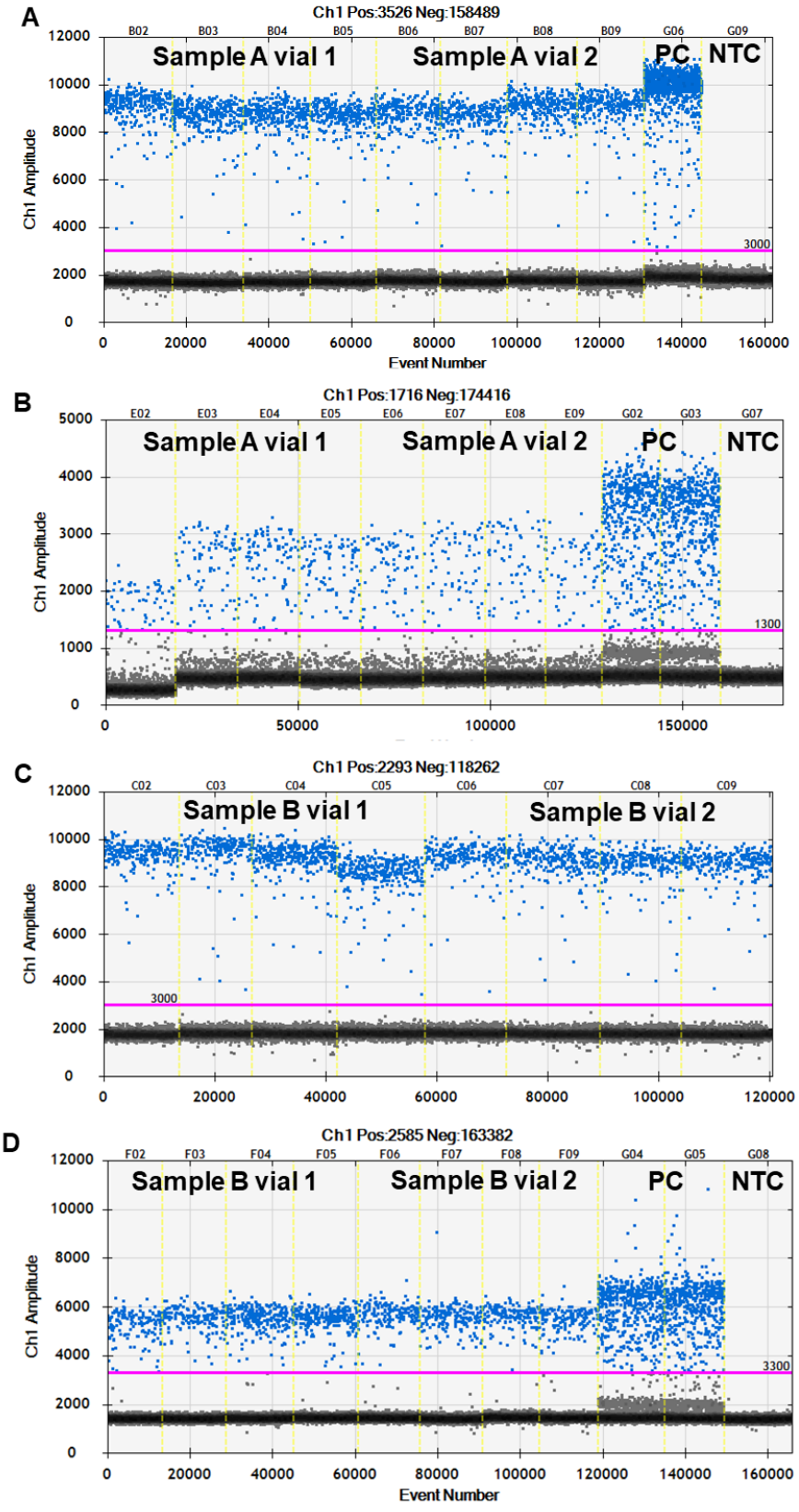


Figure 2


A
Ch1 Pos:3526 Neg:158489
B02 B03 B04 B05 B06 B07 B08 B09 G06 G09
Sample A vial 1
Sample A vial 2
PC
NTC
Ch1 Amplitude
3000
Event Number
B
Ch1 Pos:1716 Neg:174416
E02 E03 E04 E05 E06 E07 E08 E09 G02 G03 G07
Sample A vial 1
Sample A vial 2
PC
NTC
Ch1 Amplitude
1300
C
Ch1 Pos:2293 Neg:118262
C02 C03 C04 C05 C06 C07 C08 C09
Sample B vial 1
Sample B vial 2
Ch1 Amplitude
3000
D
Ch1 Pos:2585 Neg:163382
F02 F03 F04 F05 F06 F07 F08 F09 G04 G05 G08
Sample B vial 1
Sample B vial 2
PC
NTC
Ch1 Amplitude
3300
Event Number

Tables

Table 1

Target	Type of oligonucleotide	5'-sequence-3'	Final concentration in PCR (nM)	Amplicon length (bp)
UL54 [12]	UL54 forward primer	5'-GGCCTCGTAGTGAAAATTAATGGT-3'	600	72
	UL54 reverse primer	5'-GGCCGTTACTGTCTGCAGGA-3'	600	
	UL54 probe	5'-FAM-CCGTATTGGTGCGCGATCTGTTCAA-BHQ-3'	200	
A594V (adapted from [13])	A594V forward primer	5'-ACGGAGGCGTTGCTCTTTAA-3'	900	75
	A594V reverse primer	5'-GGAGCAGTGCGTGAGCTTG-3'	900	
	A594V probe	5'-FAM-CTCCAACACGCGGC-MGBNFQ-3'	300	
L595S (adapted from [13])	L595S forward primer	5'-ACGGAGGCGTTGCTCTTTAA-3'	900	75
	L595S reverse primer	5'-GGAGCAGTGCGTGAGCTTG-3'	900	
	L595S probe	5'-FAM-TTCTCCGACGCGCGG-MGBNFQ-3'	300	
M460V [8]	M460V forward primer	5'-TGTTTCATCACGACCAGTGGAA-3'	900	163
	M460V reverse primer	5'-CTGGGGTTGTACGGGTTCAC-3'	900	
	M460V probe	5'-FAM-ACGTTCACGGGTGTAA-MGBNFQ-3'	300	

Table 2

No. of cycles	Time	Temperature (°C)
1	10 min	95
45	15 sec	95
	60 sec	60
1	10 min	98
1	infinite	4

Lid temperature: 105°C
Volume: 40µL
Ramp rate: 2.5 °C/s