Applications of Digital PCR for Clinical Microbiology

Jane Kuypers, Keith R. Jerome

Department of Laboratory Medicine, University of Washington, Seattle, Washington, USA; Vaccine and Infectious Diseases Department, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA

ABSTRACT Digital PCR (dPCR) is an important new tool for use in the clinical microbiology laboratory. Its advantages over quantitative PCR (qPCR), including absolute quantification without a standard curve, improved precision, improved accuracy in the presence of inhibitors, and more accurate quantitation when amplification efficiency is low, make dPCR the assay of choice for several specimen testing applications. This minireview will discuss the advantages and disadvantages of dPCR compared to qPCR, its applications in clinical microbiology, and considerations for implementation of the method in a clinical laboratory.

KEYWORDS applications, digital PCR, quantitative PCR

Since its invention in the early 1980s, PCR has become an indispensable tool for the detection of microbiologic agents. With subsequent improvements, such as thermostable polymerases and dedicated instruments, PCR became broadly available to clinical microbiology laboratories (1). The clinical utility of PCR expanded with the development of quantitative PCR (qPCR), which enabled not only detection but also quantification of targeted nucleic acids in clinical specimens. In qPCR, the inclusion of fluorescent DNA-intercalating dyes or fluorescent dye-labeled probes within the PCR allows continuous monitoring of the amplification reaction (real-time PCR) (Fig. 1A). By measuring the PCR cycle at which fluorescence reaches a certain threshold (the threshold cycle [C_T] value) for samples with a known amount of target, a standard curve can be generated (Fig. 1B). By comparing the C_T of a clinical specimen to the standard curve, the quantity of the analyte can be calculated. The ability to quantify pathogens has proven useful as a prognostic indicator and to monitor treatment response in many infections (2).

Digital PCR (dPCR) takes a fundamentally different approach to quantifying the number of DNA molecules in a sample. As in qPCR, fluorescent dyes are included in the DNA amplification reaction. However, unlike qPCR, quantification is achieved without the need for PCR C_T values and standard curves. Instead, in dPCR, the amplification reaction is divided into thousands of independent partitions. Partitioning can be achieved by using microwell plates, capillaries, oil emulsions, or arrays. Ideally, partitioning occurs such that each individual reaction mixture contains either a single target molecule or none at all. The partitioned reactions are then amplified to the endpoint, and the number of positive (fluorescent) and negative partitions is counted (Fig. 1C and D). Based on the number of positive and negative partitions, the target copy number in the sample can be calculated (3–5). As the concentration of target increases and it becomes more likely that a given partition will contain two or more copies, Poisson's Law is used to accurately calculate the number of DNA targets per partition and the copy number in the original sample (4, 6).

In order to determine the number of DNA copies in a dPCR mixture without bias, certain conditions must be met. First, the DNA targets must be randomly distributed
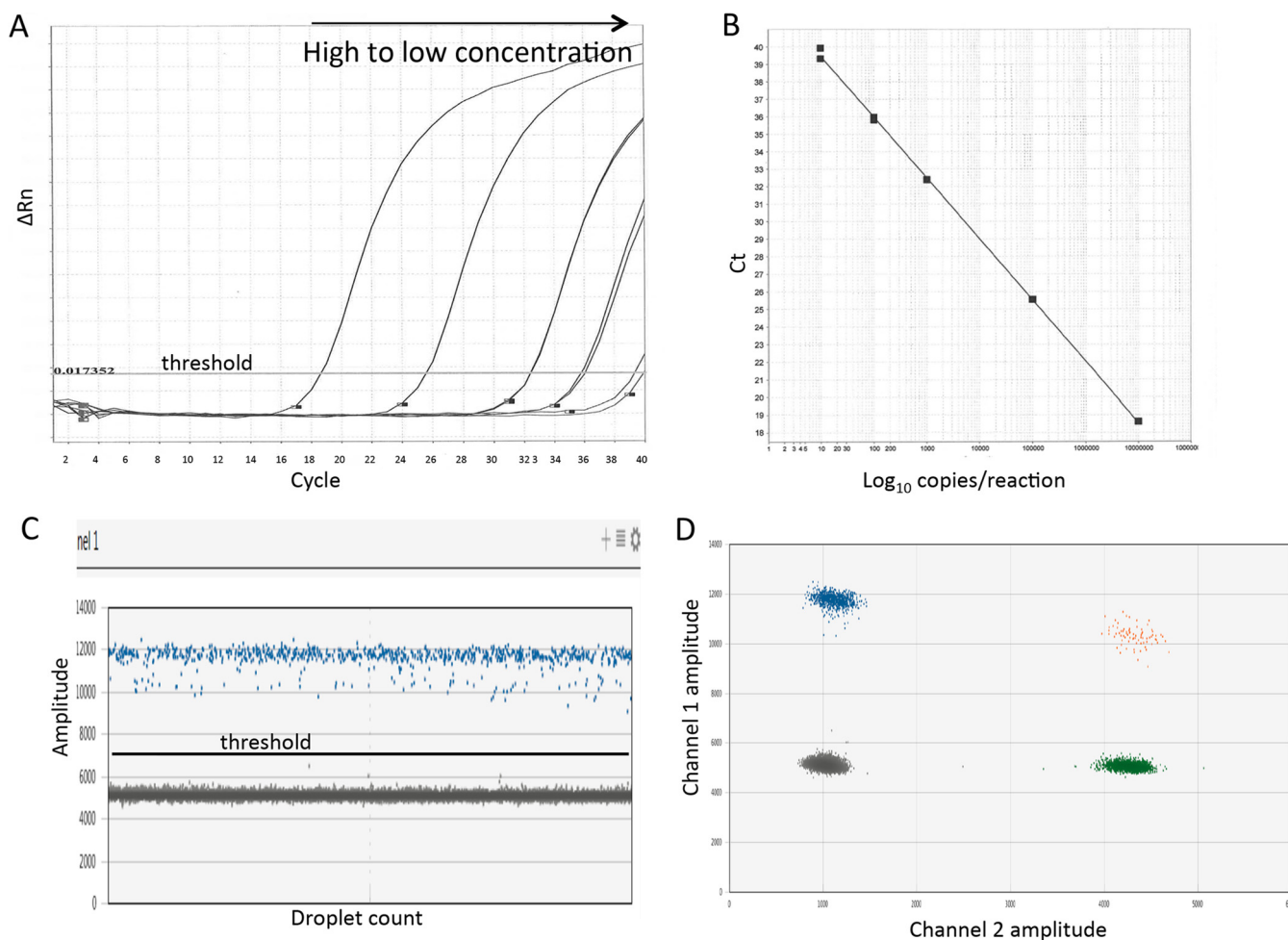
FIG 1 (A) Typical qPCR amplification plot (ΔRn [fluorescence] versus cycle) with threshold line. (B) Standard curve generated from qPCR amplification of known numbers of target (threshold cycle [Ct] versus $\log_{10}$ copies/reaction). (C) Typical dPCR droplet plot for a reaction with a single fluorophore (amplitude [fluorescence] versus droplet count) with threshold line. Droplets above the threshold line are positive, and those below are negative. The number of targets per microliter is calculated using an equation that includes the number of positive partitions, the total number of partitions, and the partition volume. (D) Two-dimensional dPCR droplet plot for a multiplex reaction (channel 1 amplitude versus channel 2 amplitude). Droplets in the bottom left corner are negative for both targets, while droplets in the top right corner are positive for both targets. Droplets in the top left and bottom right corners are positive for the channel 1 and channel 2 targets, respectively.

into the partitions, and ideally, each partition should contain no more than one target molecule. Thus, for clinical samples containing clinically relevant numbers of targets, a large number of partitions (10,000 to 100,000) is necessary to achieve the limiting dilution required for application of Poisson's Law. Samples with expected quantities of target equal to or greater than the number of partitions would require dilution to achieve accurate results. Partitions should also be of uniform size so that each will contain the same number of target molecules. Finally, amplification must be sufficiently efficient so that all partitions containing target molecules are amplified, and there must be a clear discrimination between positive and negative partitions (4, 6).

Several commercial platforms for performing digital PCR have addressed these concerns, and their availability opens up new opportunities for the use of dPCR in clinical microbiology laboratories. Current commercial dPCR platforms include the BioMark HD (Fluidigm, South San Francisco, CA), Clarity (JN Medsys, Singapore), and QuantStudio 12K Flex and 3D instruments (Thermo Fisher, Waltham, MA) that partition samples into individual reaction wells on chips and the RainDrop (RainDance, Billerica, MA) and QX100 and QX200 instruments (Bio-Rad, Hercules, CA) that partition samples using water-in-oil droplets. As with qPCR, all dPCR platforms currently require

TABLE 1 Characteristics of dPCR compared to qPCR

Advantage	Disadvantage
Absolute quantification, no standard curve	Limited reaction mixture volume
Improved interlaboratory commutability	Smaller dynamic range
Less affected by sample inhibitors	Molecular dropout
Less affected by poor amplification efficiency	Less accurate quantification of larger amplicons
More precise	Lower throughput
Better detection of low-copy-number variants	Limited multiplexing exacerbated if assay requires internal control
	More expensive instrumentation and reagents
	Higher risk for contamination
	More complex to perform

prior nucleic acid extraction of the specimen on a different instrument and optimization of PCR primer and probe design and concentration (5, 7, 8).

This minireview will discuss the advantages and disadvantages of dPCR compared to qPCR, applications for clinical microbiology, and considerations for implementation of the method in a clinical laboratory.

COMPARISON OF dPCR TO qPCR

The advantages and disadvantages of dPCR compared to qPCR are briefly summarized in Table 1. The absolute quantification of dPCR, which is achieved without reliance on a calibration curve, is a major advantage of this method (4, 6). For the relative quantification of nucleic acids performed by qPCR, the C_T value of a sample is compared to a standard curve generated by amplification of dilutions of the same template with assigned values, which must be determined by using another method. Laboratories testing the same specimens by qPCR using standard curves calibrated by different methods may obtain different copy numbers. Absolute quantification of templates in clinical specimens by dPCR, which is not dependent on calibration, provides improved accuracy and commutability of results between laboratories (3, 9). Accurate quantification by qPCR is dependent on logarithmic amplification during each PCR cycle. If amplification is inhibited by impurities in the sample or amplification efficiency is poor due to mismatches between target, primer, and probe sequences, quantification will be underestimated. Because dPCR quantifies using endpoint instead of real-time amplification, quantification is less affected by inhibitors of amplification that may be present in the sample (10, 11), and it is also less affected by poor amplification efficiency (4, 12, 13). In fact, it may be possible to perform dPCR on samples without prior extraction of the nucleic acids (14). While qPCR and dPCR are generally equally sensitive given an equivalent input of template (15), dPCR assays have been shown to quantify some targets more precisely and are especially useful for precise quantification of low viral loads for monitoring antiviral therapy (3, 4, 6, 13, 15–17). Compared to qPCR, the better precision of dPCR is also useful for detection and quantitation of rare variants (6) and for assays measuring ratios of high- and low-copy-number targets in a reaction (18). Digital PCR can detect small numbers of one target in a background of high numbers of another target, because reaction partitioning and endpoint amplification are less susceptible to competition between targets for reagents (5, 16).

However, some characteristics of dPCR may be disadvantageous compared to qPCR. These characteristics include limited sample volume per reaction, which limits the assay lower limit of detection, small dynamic range due to a limited number of partitions, and falsely low quantification due to molecular dropout, in which not all templates in a partition are amplified (3, 4) and which can happen at any copy number. This is especially a problem when quantifying RNA, because the reverse transcription step can be incomplete for some RNA targets and not all copies in a sample will be measured (19). While qPCR instruments are capable of measuring fluorescence from four to six different dyes attached to probes that bind different targets in a sample, most dPCR instruments can measure fluorescence

TABLE 2 Applications of dPCR in the clinical microbiology laboratory

Characteristic	Application
Absolute quantification without a standard curve	Quantification of pathogen load, especially targets with no available reference material
	Calibration of reference standards for qPCR
Less affected by poor efficiency of amplification	Accurate quantitation of targets with high sequence variability
Less affected by inhibitors in the sample	Accurate quantitation of samples with inhibitors
Good sensitivity and excellent precision	Precise quantitation for monitoring low pathogen loads
	Detection of rare mutations and alleles
	Reliable determination of fold change measurements
	Detection of endonuclease-mediated gene editing

from two dyes, limiting the ability for multiplex detection of different targets in the same sample. If the specific application of dPCR requires incorporation of an internal control, more-complex multiplex strategies based on differential dye concentrations or mixtures of dyes may be required (20). Other disadvantages include less accurate quantification of larger amplicons, lower specimen throughput, more expensive instrumentation and reagents, a higher risk for contamination due to the open nature of some systems for droplet preparation, and a more complex workflow that requires more hands-on time with multiple steps, which increases the time to result. Finally, even though dPCR performs absolute quantification, dPCR results using reagents and platforms from different manufacturers may not always agree. Results for quantitation of cytomegalovirus (CMV) DNA showed some variability between three sets of PCR reagents and two digital platforms (21). Many of these disadvantages are due to technical limitations that are being addressed by manufacturers who are developing improved commercial instruments and reagents (22, 23).

APPLICATIONS OF dPCR FOR CLINICAL MICROBIOLOGY

The advantages of dPCR over qPCR can be exploited by the microbiology laboratory for multiple applications (summarized in Table 2). The ability of dPCR to perform absolute quantitation that does not rely on a well-calibrated standard or highly efficient amplification is useful for the determination of pathogen loads, for quantifying targets with sequence diversity and samples with inhibitors, and for characterization of reference standards that will be used in qPCR assays. Digital PCR and reverse transcription (RT)-dPCR have been used to determine the copy numbers of DNA and RNA viruses, bacteria, and parasites in a variety of clinical specimens, including when a well-calibrated standard is not available for qPCR amplification. Assays have been used to quantify many viruses, including HIV DNA and HIV two-long terminal repeat (2-LTR) circles (13, 24), CMV (15, 17), hepatitis B virus (25), JC polyomavirus (26), human papillomavirus (27), HIV RNA (3, 6, 18), human T-lymphotropic virus (3, 6), human rhinoviruses (11), hepatitis C virus (18), hepatitis E virus (28), and human parechovirus type 3 (29). Other assays have been described for the quantification of *Mycobacterium tuberculosis* (30) and *Helicobacter pylori* (31) bacterial targets and the malaria parasite (32).

Digital PCR and RT-dPCR are especially useful for quantitation of DNA and RNA virus targets that have high sequence diversity, such as BK virus (33), human rhinovirus (12), and HIV (13). Quantitation of these viruses by qPCR and RT-qPCR is often performed using consensus primer and probe sets that are intended to detect all genotypes of the virus. However, inefficient amplification of some genotypes due to sequence mismatches between the consensus primers and probe and the target nucleic acid may lead to inaccurate quantitation (12, 33). Compared to RT-qPCR, human rhinovirus, which has a highly diverse genome, was more accurately quantified by RT-dPCR, which is less affected by amplification efficiency (12). Amplification efficiency can also be compromised by impurities in a nucleic acid sample that inhibit the PCR. Compared to

qPCR, dPCR provided more accurate quantitation of pathogens in the presence of inhibitors in the samples, including detection of cytomegalovirus in stool samples (11) and *Enterococcus* in water samples for environmental quality monitoring (34).

Another useful application of dPCR for the clinical laboratory is characterization of reference standards. Reference standards that are used in routine qPCR assays can be initially calibrated using dPCR, which does not rely on a calibrator for quantitation (6, 7, 9). Digital PCR has been employed to assign values to reference materials used in CMV qPCR assays (5, 14, 35). The National Institute of Standards and Technology recently established a new CMV standard using dPCR (36). Calibration of *Escherichia coli* plasmid standards by dPCR compared favorably to calibration by UV absorbance and mass spectrometry (37). Digital PCR has also proven valuable in the identification of copy number heterogeneity within international standards. For example, dPCR assays targeting different regions of the WHO international standards for BK and JC viruses varied in quantitation up to eightfold, depending on the region targeted. This discrepancy resulted not from imprecision in dPCR, but instead from the presence of multiple viral subpopulations within the WHO standards, a finding confirmed by next-generation sequencing (38, 39).

The relatively good sensitivity and excellent precision of dPCR make it useful for more accurate detection of low pathogen loads, for detection of minor mutations and rare allele targets, and for determining ratios of specific targets in the same sample. The better precision of dPCR reveals clinically relevant changes in viral load. Very precise quantitation of very low viral copy numbers provided more precise monitoring of residual latent HIV DNA reservoirs (13) and CMV serum viral loads for monitoring of antiviral therapy (5), although the clinical benefit of the improved precision remains to be demonstrated. Digital PCR can be used to detect very low numbers of nucleic acids circulating in blood, including DNA of infectious agents (16). Previous studies have reported detection of human papillomavirus (HPV) DNA in serum (27) and *M. tuberculosis* DNA in plasma (30). HPV DNA was present in patients with HPV-associated carcinoma (27), while a dPCR assay successfully detected *M. tuberculosis* DNA in plasma samples from patients with pulmonary tuberculosis (30).

The excellent precision of dPCR supports its application for the detection of rare point mutations in a background of wild-type sequences (5). While qPCR and pyrosequencing cannot detect less than 1 to 10% of a mutant allele in a background of nonmutated DNA, dPCR was able to detect one mutant in a background of 200,000 wild-type *KRAS* genes (40). Reported applications to clinical microbiology include detection by dPCR assays of drug resistance mutations in hepatitis C virus, *Staphylococcus aureus*, and influenza A virus (4, 6, 41). Whale et al. used dPCR for detection of oseltamivir resistance in influenza A virus and found a sensitivity as low as 0.1% of the mutation (41). This very sensitive detection of rare drug resistance sequence variants can improve patient management by facilitating a change of medication.

Another important application for dPCR includes detection of mutations induced by gene editing. Sedlak et al. described a droplet digital PCR assay that quickly quantitated a range of indel mutations in the HIV provirus with detection as low as 0.02% mutant in a wild-type background and precision (≤6% coefficient of variation [CV]) and accuracy superior to either mismatch cleavage assay or clonal sequencing compared to next-generation sequencing (42). Finally, the better precision demonstrated by dPCR affords finer fold change measurements between relevant targets. This may be particularly relevant for assays seeking to determine viral load on a per-cell basis. A good example of this is an assay for the detection of inherited chromosomally integrated human herpesvirus 6 (ciHHV-6), a multiplex dPCR assay that quantifies both human and HHV-6 DNA (18). Individuals with inherited ciHHV-6 have an HHV-6/human cell ratio of 1:1. Identification of such individuals in the context of hematopoietic cell transplantation can help in the interpretation of positive results on standard HHV-6 testing and potentially avoid unnecessary treatment with antivirals. A subsequent study describes a novel dPCR assay that identifies inherited ciHHV-6 and also determines which species,

HHV-6A or HHV-6B, is responsible for the integration in a single reaction mixture (20). In this assay, the limited multiplexing capability of dPCR was overcome by labeling the HHV-6A probe with the fluorophore 6-carboxyfluorescein (FAM) and the HHV-6B probe with a 2:1 mix of the fluorophores hexachlorofluorescein (HEX) and FAM, so that the HHV-6A- and HHV-6B-positive droplet populations sorted at different x and y amplitudes on the droplet plot.

CONSIDERATIONS FOR IMPLEMENTATION

Implementation of dPCR and RT-dPCR assays should be undertaken after some consideration. Although dPCR has several specific advantages over qPCR, dPCR is not likely to replace all qPCR assays in the clinical laboratory. The lower throughput and longer turnaround times of current dPCR systems compared to qPCR argues against their routine implementation. However, there are some applications for which dPCR will outperform qPCR and should be considered. Assays that require high precision for measurement of viral load, testing of samples that contain inhibitors of PCR amplification, amplification of targets by consensus primer and probe sets in which target diversity will lead to mismatches and loss of efficiency, and detection of rare sequences are all situations in which a dPCR assay may provide more precise and accurate results than a qPCR assay. In addition, dPCR is very useful for the characterization of reference standards for qPCR assays.

The choice of a dPCR platform is limited by the available commercial instruments and includes those that partition the reaction on physical arrays or into droplets. Early droplet-based systems generally provided more partitions than physical arrays, leading to improved sensitivity, although this distinction is less evident with newer-generation instruments. In contrast, array-based systems offer the potential of recovering individual partitions for subsequent sequencing or other analysis. Again, however, this distinction is fading as newer fluidic technologies allow the isolation of individual positive droplets. The choice of master mix may also be limited depending on the platform being used, as some instruments (particularly droplet-based systems) can be used only with specific reaction mixes. Being able to evaluate and choose the best performing reaction mix is especially important for RT-dPCR assays. One study found a high degree of variability between results when evaluating three kits for RT-dPCR (19). In addition, given a choice between a one-step RT-PCR and a two-step RT-PCR, a one-step reaction will reduce bias because the RNA will be partitioned prior to reverse transcription. Due to the variability between reaction kits, it has been suggested that a calibrator may be useful to assess RT efficiency and provide accurate quantitation of RNA by RT-dPCR (19). Similar to qPCR assay implementation, the design of PCR primers and probes must be optimized, the use of an internal control is still recommended, and all assay validation steps must be performed prior to use on clinical specimens. Useful practical advice for validation of dPCR assays can be found in the Minimum Information for Publication of Quantitative Digital PCR Experiments guidelines (digital MIQE) (43).

CONCLUSIONS

Digital PCR offers a number of clear advantages over qPCR, as outlined in this minireview. Generally, the technical disadvantages of dPCR are few, but the greater complexity and slower throughput of current dPCR platforms have served as an impediment to its incorporation into clinical laboratories. As newer-generation instruments become available that address these limitations, it is likely that dPCR will play a growing role in diagnostic laboratories.

REFERENCES

1. Bartlett JM, Stirling D. 2003. A short history of the polymerase chain reaction. Methods Mol Biol 226:3–6. https://doi.org/10.1385/1-59259 -384-4:3.

2. Mackay IM, Arden KE, Nitsche A. 2002. Real-time PCR in virology. Nucleic Acids Res 30:1292–1305. https://doi.org/10.1093/nar/30.6.1292.

3. Hall Sedlak R, Jerome KR. 2014. The potential advantages of digital PCR

for clinical virology diagnostics. Expert Rev Mol Diagn 14:501–507. https://doi.org/10.1586/14737159.2014.910456.

4. Vynck M, Trypsteen W, Thas O, Vandekerckhove L, De Spiegelaere W. 2016. The future of the polymerase chain reaction in virology. Mol Diagn Ther 20:437–447. https://doi.org/10.1007/s40291-016-0224-1.

5. Sedlak RH, Jerome KR. 2013. Viral diagnostics in the era of digital polymerase chain reaction. Diagn Microbiol Infect Dis 75:1–4. https://doi.org/10.1016/j.diagmicrobio.2012.10.009.

6. Huggett JF, Cowen S, Foy CA. 2015. Considerations for digital PCR as an accurate molecular diagnostic tool. Clin Chem 61:79–88. https://doi.org/10.1373/clinchem.2014.221366.

7. Zhang K, Lin G, Li J. 2016. Quantitative nucleic acid amplification by digital PCR for clinical viral diagnostics. Clin Chem Lab Med 54:1427–1433. https://doi.org/10.1515/cclm-2015-1101.

8. Low H, Chan SJ, Soo GH, Ling B, Tan EL. 2016. Clarity™ digital PCR system: a novel platform for absolute quantification of nucleic acids. Anal Bioanal Chem 409:1869–1875. https://doi.org/10.1007/s00216-016-0131-7.

9. Tang L, Sun Y, Buelow D, Gu Z, Caliendo AM, Pounds S, Hayden RT. 2016. Quantitative assessment of commutability for clinical viral load testing using a digital PCR-based reference standard. J Clin Microbiol 54:1616–1623. https://doi.org/10.1128/JCM.03346-15.

10. Dingle TC, Sedlak RH, Cook L, Jerome KR. 2013. Tolerance of droplet-digital PCR vs real-time quantitative PCR to inhibitory substances. Clin Chem 59:1670–1672. https://doi.org/10.1373/clinchem.2013.211045.

11. Sedlak RH, Kuypers J, Jerome KR. 2014. A multiplexed droplet digital PCR assay performs better than qPCR on inhibition prone samples. Diagn Microbiol Infect Dis 80:285–286. https://doi.org/10.1016/j.diagmicrobio.2014.09.004.

12. Sedlak RH, Nguyen T, Palileo I, Jerome KR, Kuypers J. 2017. Superiority of digital reverse transcription-PCR (RT-PCR) over real-time RT-PCR for quantitation of highly divergent human rhinoviruses. J Clin Microbiol 55:442–449. https://doi.org/10.1128/JCM.01970-16.

13. Strain MC, Lada SM, Luong T, Rought SE, Gianella S, Terry VH, Spina CA, Woelk CH, Richman DD. 2013. Highly precise measurement of HIV DNA by droplet digital PCR. PLoS One 8:e55943. https://doi.org/10.1371/journal.pone.0055943.

14. Pavsic J, Zel J, Milavec M. 2016. Digital PCR for direct quantification of viruses without DNA extraction. Anal Bioanal Chem 408:67–75. https://doi.org/10.1007/s00216-015-9109-0.

15. Sedlak RH, Cook L, Cheng A, Magaret A, Jerome KR. 2014. Clinical utility of droplet digital PCR for human cytomegalovirus. J Clin Microbiol 52:2844–2848. https://doi.org/10.1128/JCM.00803-14.

16. Hudecova I. 2015. Digital PCR analysis of circulating nucleic acids. Clin Biochem 48:948–956. https://doi.org/10.1016/j.clinbiochem.2015.03.015.

17. Hayden RT, Gu Z, Ingersol J, Abdul-Ali D, Shi L, Pounds S, Caliendo AM. 2013. Comparison of droplet digital PCR to real-time PCR for quantitative detection of cytomegalovirus. J Clin Microbiol 51:540–546. https://doi.org/10.1128/JCM.02620-12.

18. Sedlak RH, Cook L, Huang M-L, Magaret A, Zerr DM, Boeckh M, Jerome KR. 2014. Identification of chromosomally integrated human herpesvirus 6 by droplet digital PCR. Clin Chem 60:765–772. https://doi.org/10.1373/clinchem.2013.217240.

19. Sanders R, Mason DJ, Foy CA, Huggett JF. 2013. Evaluation of digital PCR for absolute DNA quantification. PLoS One 8:e75296. https://doi.org/10.1371/journal.pone.0075296.

20. Sedlak RH, Hill JA, Nguyen T, Cho M, Levin G, Cook L, Huang M-L, Flamand L, Zerr DM, Boeckh M, Jerome KR. 2016. Detection of human herpesvirus 6B (HHV-6B) reactivation in hematopoietic cell transplant recipients with inherited chromosomally integrated HHV-6A by droplet digital PCR. J Clin Microbiol 54:1223–1227. https://doi.org/10.1128/JCM.03275-15.

21. Hayden RT, Gu Z, Sam SS, Sun Y, Tang L, Pounds S, Caliendo AM. 2016. Comparative performance of reagents and platforms for quantitation of cytomegalovirus DNA by digital PCR. J Clin Microbiol 54:2602–2608. https://doi.org/10.1128/JCM.01474-16.

22. Jones GM, Busby E, Garson JA, Grant PR, Nastouli E, Devonshire AS, Whale AS. 2016. Digital PCR dynamic range is approaching that of real-time quantitative PCR. Biomol Detect Quantif 10:31–33. https://doi.org/10.1016/j.bdq.2016.10.001.

23. Shen F, Sun B, Kreutz JE, Davydova EK, Du W, Reddy PL, Joseph LJ, Ismagilov RF. 2011. Multiplexed quantification of nucleic acids with large dynamic range using multivolume digital RT-PCR on a rotational Slip-

Chip tested with HIV and hepatitis C viral load. J Am Chem Soc 133:17705–17712. https://doi.org/10.1021/ja2060116.

24. Henrich TJ, Gallien S, Li JZ, Pereyra F, Kuritzkes DR. 2012. Low-level detection and quantitation of cellular HIV-1 DNA and 2-LTR circles using droplet digital PCR. J Virol Methods 186:68–72. https://doi.org/10.1016/j.jviromet.2012.08.019.

25. Huang JT, Liu YJ, Want J, Xu ZG, Yang Y, Shen F, Liu XH, Zhou X, Liu SM. 2015. Next generation digital PCR measurement of hepatitis B virus copy number in formalin-fixed paraffin-embedded hepatocellular carcinoma tissue. Clin Chem 61:290–296. https://doi.org/10.1373/clinchem.2014.230227.

26. Giovannelli I, Ciccine N, Vaggelli G, Malva ND, Torricelli F, Rossolini GM, Giannecchini S. 2016. Utility of droplet digital PCR for the quantitative detection of polyomavirus JC in clinical samples. J Clin Virol 82:70–75. https://doi.org/10.1016/j.jcv.2016.07.008.

27. Jeannot E, Becette V, Campitelli M, Calmejane MA, Lappartient E, Ruff E, Saada S, Holmes A, Bellet D, Sastre-Garau X. 2016. Circulating human papillomavirus DNA detected using droplet digital PCR in the serum of patients diagnosed with early stage human papillomavirus-associated invasive carcinoma. J Pathol Clin Res 2:201–209. https://doi.org/10.1002/cjp2.47.

28. Nicot F, Cazabat M, Lhomme S, Marion O, Saune K, Chiabrando J, Dubois M, Kamar N, Abravanel R, Isopet J. 2016. Quantification of HEV RNA by droplet digital PCR. Viruses 8:E233. https://doi.org/10.3390/v8080233.

29. Aizawa Y, Koyama A, Ishihara T, Onodera O, Saitoh A. 2016. Performance of a real-time PCR-based approach and droplet digital PCR in detecting human parechovirus type 3 RNA. J Clin Virol 84:27–31. https://doi.org/10.1016/j.jcv.2016.09.009.

30. Ushio R, Yamamoto M, Nakashima K, Watanabe H, Nagai K, Shibata Y, Tashiro K, Tsukahara T, Nagakura H, Horita N, Sata T, Shinkai M, Kudo M, Ueda A, Kaneko T. 2016. Digital PCR assay detection of circulating Mycobacterium tuberculosis DNA in pulmonary tuberculosis patient plasma. Tuberculosis (Edinb) 99:47–53. https://doi.org/10.1016/j.tube.2016.04.004.

31. Talarico S, Safaeian M, Gonzalez P, Hildesheim A, Herrero R, Porras C, Cortes B, Larson A, Fang FC, Salama NR. 2016. Quantitative detection and genotyping of Helicobacter pylori from stool using droplet digital PCR reveals variation in bacterial loads that correlates with cagA virulence gene carriage. Helicobacter 21:325–333. https://doi.org/10.1111/hel.12289.

32. Koepfli C, Nguitragool W, Hofmann NE, Robinson LJ, Ome-Kaius M, Sattabongkot J, Felger I, Mueller I. 2016. Sensitive and accurate quantification of human malaria parasites using droplet digital PCR (ddPCR). Sci Rep 6:39183. https://doi.org/10.1038/srep39183.

33. Hoffman NG, Cook L, Atienza EE, Limaye AP, Jerome KR. 2008. Marked variability of BK virus load measurement using quantitative real-time PCR among commonly used assays. J Clin Microbiol 46:2671–2680. https://doi.org/10.1128/JCM.00258-08.

34. Wang D, Yamahara KM, Cao Y, Boehm AB. 2016. Absolute quantification of enterococcal 23S rRNA gene using digital PCR. Environ Sci Technol 50:3399–3408. https://doi.org/10.1021/acs.est.5b05747.

35. Hayden RT, Gu Z, Sam SS, Sun Y, Tang L, Pounds S, Caliendo AM. 2015. Comparative evaluation of three commercial quantitative cytomegalovirus standards by use of digital and real-time PCR. J Clin Microbiol 53:1500–1505. https://doi.org/10.1128/JCM.03375-14.

36. Haynes RJ, Kline MC, Toman B, Scott C, Wallace P, Butler JM, Holden MJ. 2013. Standard reference material 2366 for measurement of human cytomegalovirus DNA. J Mol Diagn 15:177–185. https://doi.org/10.1016/j.jmoldx.2012.09.007.

37. Liang W, Xu L, Sui Z, Li Y, Li L, Wen Y, Li C, Ren S, Liu G. 2016. Quantification of plasmid DNA reference materials for Shiga toxin-producing Escherichia coli based on UV, HR-ICP-MS and digital PCR. Chem Cent J 10:55. https://doi.org/10.1186/s13065-016-0201-0.

38. Bateman AC, Greninger AL, Atienza EE, Limaye AP, Jerome KR, Cook L. 2017. Quantification of BK virus standards by quantitative real-time PCR and droplet digital PCR is confounded by multiple virus populations in the WHO BKV international standard. Clin Chem 63:761–769. https://doi.org/10.1373/clinchem.2016.265512.

39. Greninger AL, Bateman AC, Atienza EE, Wendt S, Makhsous N, Jerome KR, Cook L. 2017. Copy number heterogeneity of JC virus standards. J Clin Microbiol 55:824–831. https://doi.org/10.1128/JCM.02337-16.

40. Pekin D, Skhiri Y, Baret JC, Le Corre D, Mazutis L, Salem CB, Millot F, El Harrak A, Hutchison JB, Larson JW, Link DR, Laurent-Puig P, Griffiths AD, Taly V. 2011. Quantitative and sensitive detection of rare mutations

using droplet-based microfluidics. Lab Chip 11:2156–2166. https://doi
.org/10.1039/c1lc20128j.

41. Whale AS, Bushell CA, Grant PR, Cowen S, Gutierrez-Aguirre I, O'Sullivan
DM, Zel J, Milavec M, Foy CA, Nastouli E, Garson JA, Huggett JF. 2016.
Detection of rare drug resistance mutations by digital PCR in a human
influenza A virus model system and clinical samples. J Clin Microbiol
54:392–400. https://doi.org/10.1128/JCM.02611-15.

42. Sedlak RH, Liang S, Niyonzima N, De Silva Feelixge HS, Roychoudhury P,
Greninger AL, Weber ND, Boissel S, Scharenberg AM, Cheng A, Magaret

A, Bumgarner R, Stone D, Jerome KR. 2016. Digital detection of endo-
nuclease mediated gene disruption in the HIV provirus. Sci Rep 6:20064.
https://doi.org/10.1038/srep20064.

43. Huggett JF, Foy CA, Benes V, Emslie K, Garson JA, Haynes R, Helle-
mans J, Kubista M, Mueller RD, Nolan T, Pfaffl MW, Shipley GL,
Vandesompele J, Wittwer CT, Bustin SA. 2013. The digital MIQE
guidelines: minimum information for publication of quantitative dig-
ital PCR experiments. Clin Chem 59:892–902. https://doi.org/10.1373/
clinchem.2013.206375.