

Mini Review

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Quantitative nucleic acid amplification by digital PCR for clinical viral diagnostics

DOI 10.1515/cclm-2015-1101

Received November 10, 2015; accepted December 27, 2015; previously published online February 4, 2016

Abstract: In the past few years, interest in the development of digital PCR (dPCR) as a direct nucleic acid amplification technique for clinical viral diagnostics has grown. The main advantages of dPCR over qPCR include: quantification of nucleic acid concentrations without a calibration curve, comparable sensitivity, superior quantitative precision, greater resistance to perturbations by inhibitors, and increased robustness to the variability of the target sequence. In this review, we address the application of dPCR to viral nucleic acid quantification in clinical applications and for nucleic acid quantification standardization. Further development is required to overcome the current limitations of dPCR in order to realize its widespread use for viral load measurements in clinical diagnostic applications.

Keywords: digital PCR; nucleic acid; quantification; standardization; virus.

Introduction

Virus infections are one of the major causes of various serious diseases and therefore represent a significant global healthcare burden. For instance, the cumulative effect of hepatitis B virus (HBV) infections is related to hepatocellular carcinoma (HCC) [1] and 20% of hepatitis C virus (HCV) infections progress to chronic hepatitis and cirrhosis [2]. For immunocompromised patients, including organ transplant patients or HIV-infected patients, human cytomegalovirus (CMV) infection can lead to pneumonia, hepatitis, gastroenteritis, etc. [3]. Human T cell

lymphotropic virus 1 (HTLV-1), the first discovered oncogenic human retrovirus and a member of the retroviridae family, causes adult-T cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [4]. Therefore, reliable quantification of viral load in clinical samples is important for the following reasons: to fully understand the clinical relevance of the virus, to monitor the efficacy of antiviral therapy, and to assist in determining changes to the therapeutic regimen.

In the past few years, numerous quantitative nucleic acid amplification methods have been used to quantify viral DNA or RNA loads in clinical samples. These methods include quantitative or real-time polymerase chain reaction (qPCR), nucleic acid sequence-based amplification (NASBA), branched DNA (bDNA) assays and developing real-time transcription-mediated amplification (TMA). Among these methods, qPCR is the most widely used for diagnosing viral disease from clinical samples [5]. First described in the 1990s, digital PCR (dPCR) is a relatively new technique [6]; however, it has been recently developed into a commercially viable option to determine the absolute quantity of target nucleic acid in clinical applications, including for oncology, infectious disease, fetal genetic screening and in predicting transplant rejection [7]. Digital PCR has potential advantages for clinical virology diagnostics, although more data are required to fully assess its application to viral DNA and RNA load testing. In this review, we provide an overview of the current state of quantitative nucleic acid amplification by dPCR for clinical viral diagnostics, the application of dPCR to clinical detection standardization, and its current limitations in clinical virus quantification.

Comparison of qPCR and dPCR

In qPCR, during the exponential amplification phase, the fluorescent signal of a sequence-specific fluorescent probe or dye bound to double-stranded DNA can be measured once it crosses a minimum threshold of detection. The concentration of nucleic acid in the sample is

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inversely related to the cycle threshold (Ct) [5]. In qPCR, the nucleic acid concentration of the sample is indirectly measured, and this measurement depends on the relationship of Ct to a calibration curve consisting of known standards. Variation in calibration standards may contribute to non-reproducible results and a general lack of commutability within and/or between laboratories [7, 8]. Digital PCR (dPCR) is a direct nucleic acid amplification technique (NAAT). Like qPCR, dPCR uses sequence-specific primers to amplify target template DNA. In addition, samples are prepared and nucleic acids extracted by similar methods for both qPCR and dPCR. The key difference between qPCR and dPCR is that amplification in dPCR is carried out in individual bulk partitions [7]. The readout of dPCR is numbers of (nucleic acid) molecules. Using dPCR, the nucleic acid concentration (i.e. copy number per microliter) in the original patient sample can be estimated by poisson statistics and the following equation: copy number per microliter = $-(\text{dilution factor/partition or droplet volume}) \times (1 - \text{the fraction of positive reaction})$ [9]. When detected by commercial kits, quantitative results of qPCR are usually reported as IU/mL, such as for HCV, HBV, CMV, or EBV, a standard practice that can be traced back to WHO standards [5]. On the other hand, quantitative results of dPCR are usually expressed as copy numbers. It should be noted that a universal conversion factor between IU and copy numbers does not exist, and different detection platforms and reagents might use different conversion factors [10]. An example of an IU-to-copy number conversion factor was calculated for CMV load detection utilizing WHO standards [11].

Compared to qPCR, dPCR has several potential advantages. The major strength of dPCR is that it directly quantifies the absolute copy numbers of nucleic acids (i.e. does not rely on a calibration curve or Ct value) [12]. Digital PCR has comparable sensitivity with qPCR, exhibits superior precision, is more resistant to inhibition, and is more robust to the variability of the target sequence. However, there are still numerous limitations to the widespread adoption of dPCR including: lower throughput, poor scalability, longer turnaround time, increased risk of contamination, error introduced during sample partitioning, systematic bias leading to underestimation, lower linear dynamic range, problems with sample overload, higher cost, etc. [7, 12].

Digital PCR workflow and platforms

At least six different commercialized dPCR platforms are currently available [9]. The key difference among these

platforms lies in the method of reaction partitioning, which is generally achieved either on chip or through water-in-oil emulsions or droplets [13]. The “chip dPCR” platforms (termed cdPCR) include BioMark HD from Fluidigm, QuantStudio 12K Flex instrument, and 3D dPCR from Life Technologies. The “chip dPCR” platform makes use of chips perforated with through-wells, and the sample is delivered into individual reaction wells. Bio-Rad laboratories and RainDance offer “droplet dPCR” (termed ddPCR)-format-based dPCR systems: QX100 and QX200 from Bio-Rad and RainDrop from RainDance [9]. In ddPCR systems, diluted samples are divided into thousands or millions of water-in-oil droplets. Both formats can use the same patient samples, DNA extraction methods, primers, and probes. Different dPCR formats, in addition to the two mentioned above, have also been investigated; therefore, Huggett et al. [12] advocate using only the term “dPCR” to avoid any confusion. A comparison of the workflow for cdPCR and ddPCR platforms is shown in Figure 1.

Clinical application of dPCR in viral DNA quantification

Several studies have utilized dPCR to directly quantify DNA viremia loads in clinical samples. Hayden et al. used dPCR for CMV load testing of standards and 50 human plasma samples [8]. In their study, dPCR accurately measured CMV load data and showed less variability than qPCR at higher concentrations (i.e. at $4 \log_{10}$ copies/mL) of WHO standards; however, dPCR was less sensitive than qPCR. Sedlak and colleagues showed that the sensitivity of dPCR could be improved by increasing the input volume of CMV DNA from patient plasma samples [11]. They found that increasing the input volume of template DNA rendered the sensitivity of dPCR equivalent to that of standard clinical qPCR. Consistent with results from the study by Hayden et al., dPCR showed less variability than qPCR at viral loads $\geq 4 \log_{10}$ copies/mL, and results from the two studies correlated well for all patient samples. Previous studies [14, 15] of CMV DNA testing have also demonstrated that dPCR exhibits increased tolerance to the presence of inhibitory chemicals. Inhibitors, including SDS, heparin [15], EDTA [14, 15], pooled mixed human plasma, and ethanol [14], were directly introduced into CMV DNA PCR reactions. dPCR was more tolerant than qPCR to the presence of most of these inhibitors, with the exception of EDTA. Differences in the effects of inhibitors might

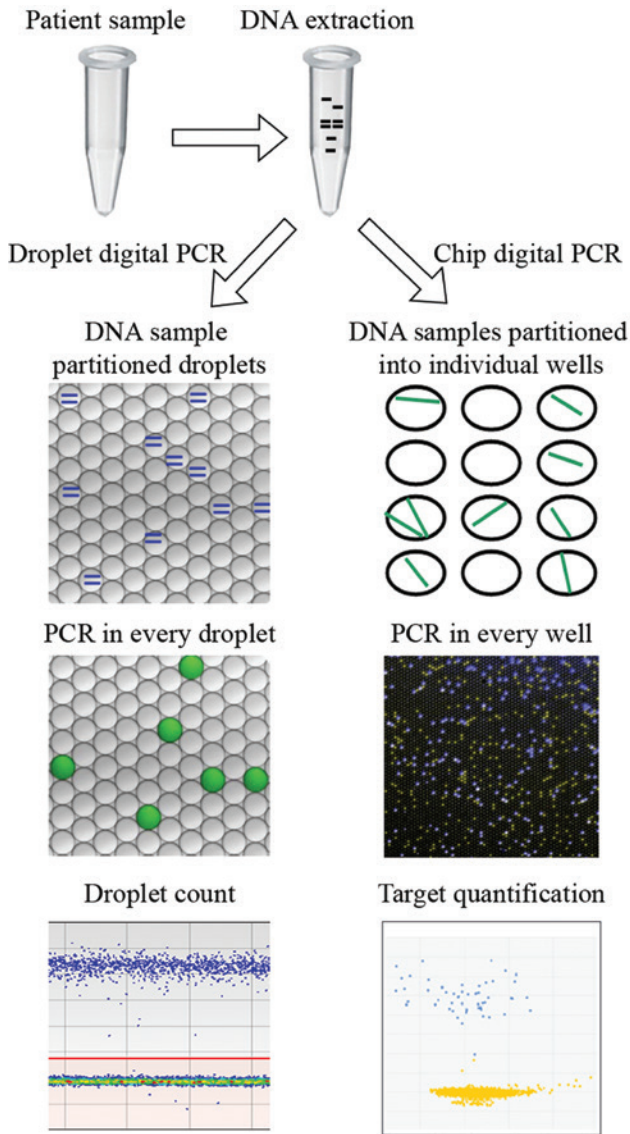


Figure 1: Digital PCR workflow.

Both formats can begin with the same patient samples and DNA extraction methods. The same primers and probes can be used for amplification. The key difference between “chip dPCR” and “droplet dPCR” lies in the methods of reaction partitioning: by plate format or by water-in-oil emulsion, respectively. Absolute DNA copy number per microliter is directly calculated.

result from differences in their mechanism of inhibition. However, an internal positive control is still necessary as the measured quantitative value might be reduced by the inhibitors [14].

The second application of dPCR in viral DNA quantification is for the diagnosis of HBV. Huang et al. [16] measured HBV copy numbers in 131 formalin-fixed paraffin-embedded (FFPE) HCC tissues of different tumor stages using dPCR. HBV copy numbers ranged from 1.1

to 175.5 copies/ μL in their study. They demonstrated that dPCR could sensitively and specifically measure low HBV viral loads, down to the level of a single molecule, in clinical FFPE samples. Positive correlations between the copy number of HBV and serum cholinesterase and liver tissue covalently closed circular HBV DNA (cccDNA) and the development of HCC were observed, indicating that dPCR might be used in the early detection of HCC and in assessing the efficacy of HBV therapy and liver transplantation. In addition, dPCR could be used to accurately and sensitively quantify HBV cccDNA in HepG2.215 lysate samples, indicating that dPCR could be used in the future to monitor the fluctuation of cccDNA during the course of antiviral therapy [17]. However, it should be noted that dPCR was less sensitive than qPCR in measuring low HBV DNA levels (<15 IU/mL) in 29 plasma samples from blood donors [18]; therefore, further optimization of dPCR for detecting HBV in clinical serum samples is still required.

Levels of total HIV-1 DNA, integrated HIV-1 DNA (provirus), and unintegrated HIV-1 DNA, i.e. 2-long terminal repeat (LTR) circles, provide information about persistent HIV infection [19]. However, HIV DNA, at low levels, is not easily quantifiable by qPCR because of decreased accuracy and exponentially amplifying noise [20]. The third successful application of dPCR in viral DNA quantification is measuring total HIV DNA and 2-LTR circles in samples from clinical patients [21, 22]. Henrich et al. [21] found that dPCR measured $\sim 10\%$ – 60% fewer DNA copies than qPCR, but the sensitivity of detection between the two methods was comparable. Low levels of 2-LTR circles (viral load from 203 to 780 copies/mL) could be detected in the samples of three patients by both dPCR and qPCR, but the sample number was limited. Analysis of over 150 peripheral blood mononuclear cells (PBMC) clinical samples by dPCR and by qPCR [21] demonstrated that pol copy numbers for total HIV-1 DNA were significantly correlated between the two methods. 2-LTR copy numbers measured by dPCR and qPCR were also significantly but weakly correlated. The average coefficient of variation was 4-fold lower for pol (total HIV-1 DNA) and 20-fold lower for 2-LTR by dPCR, indicating an improvement in accuracy. On the other hand, high variability in HIV genomes exists among patients; therefore, a two-stage touchdown dPCR strategy could be performed. Total DNA was isolated from PBMC samples of 16 HIV-infected patients and the results illustrated that touchdown dPCR strategy can effectively measure HIV-1 DNA. However, on qPCR platforms, a touchdown strategy is not easily implemented because it reduces reaction efficiency and interferes with absolute quantification [23].

Clinical application of dPCR in viral RNA quantification

Quantification of viral RNA is important for monitoring the progress of viral infections and measuring viral RNA load at regular intervals after treatment to determine the efficacy of treatment. Real time quantitative reverse transcription real-time PCR (RT-qPCR) is the most widely used method for quantifying RNA of HIV, HCV and other viruses [10]. Recently, several studies have used RT-dPCR for RNA virus quantification, including for the quantification of cell-associated (CA) HIV-1 RNA, a potential virological biomarker of antiretroviral therapy (ART). Kiselinova et al. [24] compared dPCR and seminested qPCR results for the quantification of CA HIV-1 RNA in 34 PBMC samples. Unspliced (us) (34 samples) and multiply spliced (ms) (23 samples) CA HIV-1 RNA quantification was also performed. A similar percentage of usRNA samples (91%) was detected by both methods; however, ms RNA samples could be detected more frequently with dPCR (61%) than with seminested qPCR (39%). A correlation between these two methods was observed. However, false-positive signals were observed using dPCR, which warrants further study.

When using RT-dPCR, it is important to consider sample partitioning. There are two protocols for RT-dPCR: two-step and one-step. In two-step RT-dPCR protocols, RNA is reverse-transcribed to cDNA before samples are partitioned; therefore, two-step RT-dPCR quantifies absolute numbers of target cDNA. If the cDNA copy number is not linearly correlated with the initial target RNA copy number, significant bias may occur. One-step RT-dPCR protocols may reduce this bias because sample partitioning of RNA molecules is performed prior to RT [25]. In a previous study [24], two-step RT-dPCR protocol was performed. To more accurately quantify RNA, cDNA copy number was converted to RNA copy number based on a standard curve; therefore, RNA quantification was indirectly determined, as is done with qPCR. One-step RT-dPCR was used to quantify waterborne RNA virus (rotavirus) in water samples, providing an absolute quantification of viral RNA copies without a calibration curve [26].

One limitation of dPCR is its lower dynamic range than qPCR due to the number of partitions, which does not meet clinical requirements for measuring RNA concentration. Shen et al. [10] have designed a multivolume digital RT-PCR platform on a microfluidic rotational Slip-chip to quantify HIV and HCV viral loads. The dynamic range of analysis for a single sample was improved to 1.7×10^2 – 2.0×10^7 molecules/mL, and the limit of detection

was 40 molecules/mL. Using the design, no cross-contamination was observed. Two HIV patients' viral RNA results agreed well with those detected by the Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 test. Therefore, a multivolume digital RT-PCR platform was useful for precise viral HIV and HCV RNA quantification and could be potentially valuable for monitoring other viral loads.

Other applications of dPCR for clinical virus diagnosis

Another application of dPCR includes viral DNA ratiometric assays, including for chromosomally integrated (ci) viral genomes. Human herpesvirus 6 (HHV-6) integration into chromosome telomere regions occurs in about 1% of the population. Using qPCR, ratiometric assays for HHV-6 are problematic because they detect both HHV-6 infection and ciHHV-6 [27, 28]. A previous study [28] accurately identified the ratio of HHV-6 and cellular DNA by developing a duplex dPCR for the quantitation of HHV-6 and human genome copies. Using patient buffy coat samples confirmed by fluorescence in situ hybridization (FISH), a precise ratio near 1 HHV-6/cell was measured by dPCR, which demonstrated the potential application of dPCR for detecting ciHHV-6. Using dPCR, ciHHV-6 could be detected in stored plasma samples with 100% sensitivity and 82% specificity. It should be noted that plasma sample testing is used for screening testing because of the low positive predictive value (5%–8%) in the general population, and buffy coat cells are the optimal specimen type for clinical testing. Leibovitch et al. [29] identified two PBMC samples from a set of healthy donor PBMC samples with possible ciHHV-6 using duplexed or triplexed dPCR. Both ciHHV-6A and ciHHV-6B could be detected by dPCR. This study also first designed a multiplex dPCR by using probes specific for HHV-6A and HHV-6B in identifying coinfection of HHV-6A and HHV-6B in healthy donor samples and individuals with multiple sclerosis (MS), which demonstrated that dPCR was suitable for precisely detecting viral coinfection with low levels of HHV-6A and HHV-6B DNA.

High cerebrospinal fluid (CSF) to PBMC cell proviral load (PVL) ratio of HTLV-1, or the frequency of infected cells, is useful in clinical diagnosis of HTLV-1 infected individuals. One study evaluated dPCR methods for HTLV-1PVL quantification [30], showing that dPCR was a precise and reproducible method for the detection of HTLV-1 in CSF samples with low cell numbers. The coefficient of variation was lower than that of qPCR. Additionally, the amplitudes of positive droplets for two samples were lower

than other samples. Sequence analysis of the target region demonstrated point mutations, suggesting that changes in the amplitude of signals may indicate the occurrence of mutations in the target sequence. Therefore, the study demonstrated a potential application of dPCR for detecting a virus mutation. In fact, the application of dPCR to detect rare mutants in a background highly enriched with the wild type sequence has been widely used in oncology, such as for the *KRAS* oncogene and *EGFR* mutations in tumor tissue. However, its application for the detection of mutations in polymorphic viral genomes is rare [27]. High-throughput and sensitive quantitation of a hepatitis C virus mutation at amino acid 70 in serum samples by dPCR has been recently reported [2]. The mutation may influence antiviral treatment efficacy. The linear range of dPCR is from 2.5 to 10^5 copies, and the limit of detection (LOD) of mutants was 0.005% with a 20,000-fold excess of the wild type sequence. Though dPCR assays could potentially be used for viral mutation detection, it should be noted that dPCR is probably suitable for the quantification of mutations only when there are few variant sequences near the target sequence.

Application of dPCR for quantification standardization

Inter-laboratory variability in NAAT was often observed in proficiency testing schemes, which may arise for various reasons: variable methods for nucleic acid extraction, variable methods for performing qPCR, variable calibration reagents, and the use of different platforms and reagents [31]. To improve the consistency and to reduce the

variability of quantification for viral loads, developing higher order reference materials and standards is important. The manufacturer's calibrators could be traced to these higher-order reference materials and standards. The WHO has released international standards for HIV, HCV, CMV and EBV, and adoption of these standards could improve the consistency of quantitative viral load testing [3]; however, values of these standards were established based on an average value from a collaborative study and were reported in conventional units (IU) [7]. Digital PCR is considered a potential higher-order reference measurement method for virus NAAT because it directly measures the absolute numbers of molecules and does not rely on calibration [3]. In recent years, dPCR has been used to assign values for reference materials, and nucleic acid concentrations were expressed as copy numbers. For example, the National Institute for Standards and Technology (NIST) has created a standard reference material (SRM 2366) for the measurement of human CMV DNA, which was assigned values by dPCR with the concentration unit of copies/ μ L [31]. The quantitative relationship of copies/mL and IU/mL using WHO standards for CMV, based on an international consensus, was 1.5 copies/IU [11]. Four dPCR platforms were comparably accurate in quantifying DNA copy number of a certified plasmid DNA reference material, and the study may provide some reference information for the establishment of a reference material [9]. Furthermore, dPCR has been used to determine the copy number concentration of a certified plasmid reference material (ERM-AD 623) for BCR-ABL1 mRNA quantification [32]. A certified reference material can be used by manufacturers and laboratories to improve the quality of nucleic acid quantification, and dPCR technology offers a reliable method for establishing calibration reagents.

Table 1: The utilities of dPCR for viral diagnostics using clinical samples in some studies.

Virus	Study marker	Sample type	Sample number	Instrument	References
HBV	DNA	FFPE	107	Bio-Rad QX100	[16]
HBV	DNA	Cell line	–	Bio-Rad QX100	[17]
HBV	DNA	Plasma	29	Bio-Rad QX100	[18]
CMV	DNA	Plasma	50	Bio-Rad	[8]
CMV	DNA	Plasma	85	Bio-Rad	[11]
CMV	DNA	Plasma	20	Fluidigm 12,765 Digital Array Chips	[14]
HIV	total DNA and 2-LTR	PBMC	7/3	Bio-Rad	[21]
HIV	total DNA and 2-LTR	PBMC	156	Bio-Rad QX100	[22]
HIV	Cell-associated HIV-1 RNA	PBMC	34	Bio-Rad QX100	[24]
HIV	total DNA	PBMC	16	Bio-Rad QX100	[23]
HHV-6	DNA	Buffy coat, plasma, tissue	68	Bio-Rad QX100	[28]
HHV-6	DNA	PBMC, serum	169	Bio-Rad QX100	[29]
HTLV-1	DNA	CSF, PBMC	50	Bio-Rad QX100	[30]
HCV	Mutation	Serum	87	Bio-Rad QX100	[2]

Perspective on dPCR in clinical virus quantification

The applications of dPCR for viral diagnostics in previous studies are summarized in Table 1. These studies have shown that dPCR can be used to quantify nucleic acids with improved accuracy and precision. However, dPCR is an emerging technology with conceptual advantages; however, its current limitations compared with qPCR limit its widespread adoption for clinical viral diagnostics. Therefore, dPCR technologies requires further development to realize its use in clinical diagnostic for viral load measurement. Finally, it should be noted that next-generation sequencing is developing rapidly as the main competitor of PCR-based technology, and the future application of dPCR in molecular diagnostics remains to be determined.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: This work was supported by the National High Technology Research and Development Program (863 Program) (2011AA02A116) and the National Natural Science Foundation of China (81201347).

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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