Diagnostic utility of droplet digital PCR for HIV reservoir quantification

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Abstract

Quantitative real-time PCR (qPCR) is implemented in many molecular laboratories worldwide for the quantification of viral nucleic acids. However, over the last two decades, there has been renewed interest in the concept of digital PCR (dPCR) as this platform offers direct quantification without the need for standard curves, a simplified workflow and the possibility to extend the current detection limit. These benefits are of great interest in terms of the quantification of low viral levels in HIV reservoir research because changes in the dynamics of residual HIV reservoirs will be important to monitor HIV cure efforts.

Here, we have implemented a systematic literature screening and text mining approach to map the use of droplet dPCR (ddPCR) in the context of HIV quantification. In addition, several technical aspects of ddPCR were compared with qPCR: accuracy, sensitivity, precision and reproducibility, to determine its diagnostic utility.

We have observed that ddPCR was used in different body compartments in multiple HIV-1 and HIV-2 assays, with the majority of reported assays focusing on HIV-1 DNA-based applications (i.e. total HIV DNA). Furthermore, ddPCR showed a higher accuracy, precision and reproducibility, but similar sensitivity when compared to qPCR due to reported false positive droplets in the negative template controls with a need for standardised data analysis (i.e. threshold determination).

In the context of a low level of detection and HIV reservoir diagnostics, ddPCR can offer a valid alternative to qPCR-based assays but before this platform can be clinically accredited, some remaining issues need to be resolved.

Keywords: droplet digital PCR, digital PCR, quantitative real-time PCR, HIV quantification, virus detection

Introduction

In the last two decades, quantitative real-time PCR (qPCR) has become a standard tool in many molecular laboratories for the quantification of viral nucleic acids and rigorously implemented in accredited clinical testing [1]. Despite its success, qPCR remains an indirect quantification method that relies on pre-validated standard curves, and is susceptible to small changes in reaction efficiency, which can substantially bias quantification due to the logarithmic nature of a PCR reaction. In this context, digital PCR (dPCR) provides an alternative type of technology that benefits from direct quantification without the need for a standard curve, and is associated with a better tolerance to small changes in PCR efficiency [2,3].

The concept of dPCR-based quantification, initially called single molecule PCR, was described before the invention of qPCR [4]. When using dPCR, a sample is divided into multiple partitions, each representing an isolated end-point PCR reaction. In a sufficiently diluted sample (at a limiting dilution), the distribution of the template molecules within these partitions follows a Poisson distribution. Consequently, this distribution can be used to deduce the concentration of target nucleic acids in a sample from the frequency of positive to negative partitions in the dPCR [5–7].

Notwithstanding the straightforward principle of dPCR, the high costs and labour-intensive procedures to generate high numbers of PCR partitions had hampered a wider use of dPCR until recently [8]. Thanks to technological developments in microfluidics, a series of digital PCR platforms has been developed that can generate high numbers of PCR partitions per sample at a reasonable price [9]. However, as with all new technologies, extensive optimisation and validation are required to assess whether the newer technology offers an improved platform for nucleic acid quantification and is able to provide additional benefits.

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The advantages of such technology are especially promising in the setting of HIV reservoir and cure research because the quantification of HIV reservoirs by PCR becomes challenging when addressing small reservoir sizes and subtle changes in reservoir dynamics [10]. Therefore, this review explores and compares dPCR with qPCR applications in the field of HIV research in particular. It also focuses on droplet digital PCR (ddPCR), as this is currently the most frequently used dPCR platform (Figure 1).

Materials and methods

Systematic literature screen

We performed a systematic literature search of ddPCR in the context of HIV quantification using the PubMed (www.ncbi.nlm.nih.gov/pubmed) and ISI Web of Science databases. The following search terms were used to construct the initial dataset of articles: 'HIV', 'polymerase chain reaction' and 'PCR'. Next, only research articles and English-language manuscripts were considered for the period between November 2011 (first report on ddPCR) and March 2016. In addition, the use of ddPCR was also searched for in clinical trials related to HIV intervention studies with published articles (on clinicaltrials.gov). Therefore, the following search term was used: 'HIV reservoir', with additional criteria: interventional studies received from November 2011 until March 2016. Subsequently, all articles were pooled together, duplicates were removed and text mining was performed within the available full text articles using the following search terms: 'droplet digital PCR' and 'ddPCR' (Appendix 1). The remaining set of articles was manually evaluated with the following exclusion criteria: (1) ddPCR was used for the quantification of other assays than HIV; (2) only HIV qPCR was performed; (3) review articles; (4) descriptive data analysis method; and (5) HIV quantification was not performed on patient samples (Appendix 2).

Evaluation criteria for the comparison of ddPCR and qPCR

The ddPCR and qPCR platforms were compared in terms of their different technical aspects, when data was available (Table 1).
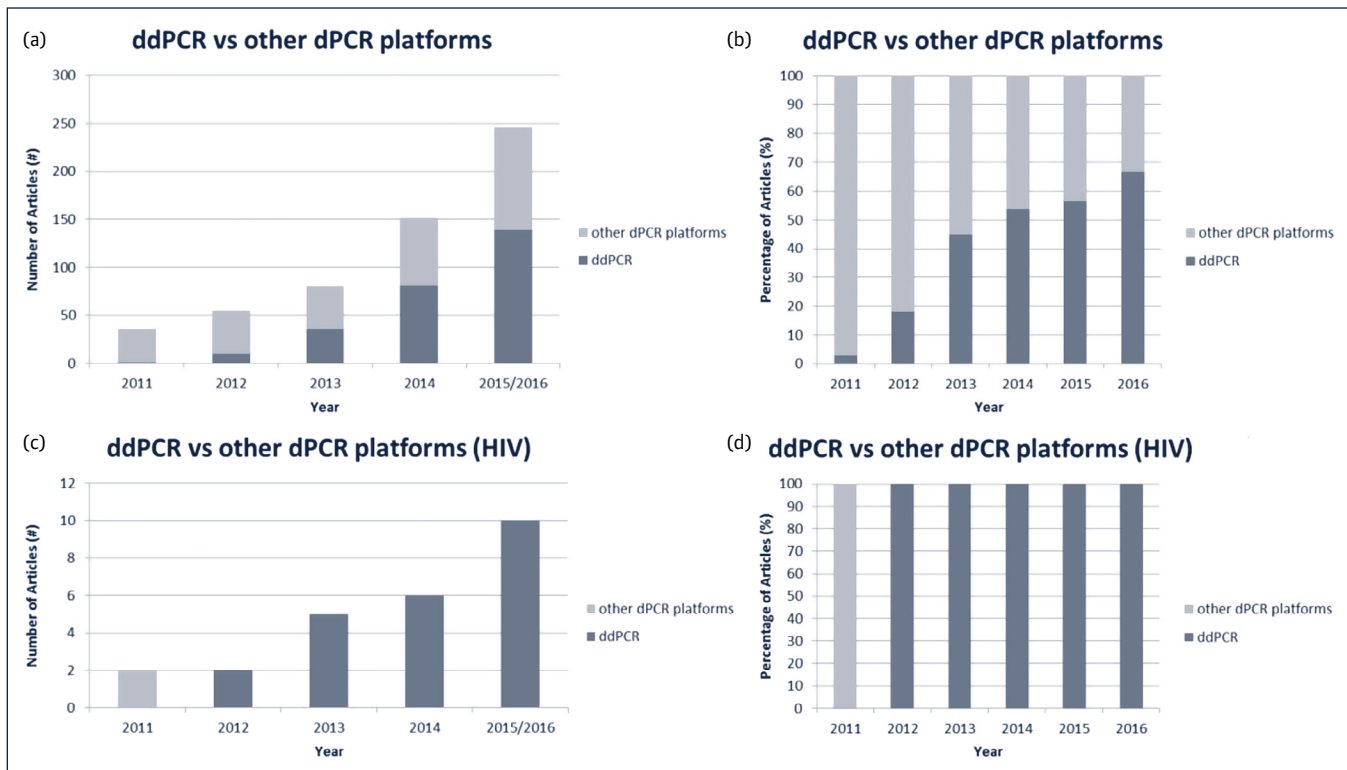
Figure 1. Reported use of different digital PCR platforms over time in published articles from November 2011 until March 2016. The absolute numbers (a, c) and ratios in percentage (b, d) are depicted for ddPCR *vs* non-ddPCR usage in a general dPCR context (a, b) and in an HIV-specific context (c, d)

Table 1. Overview of technical aspects used for comparison of ddPCR and qPCR

Technical aspect	Definition
Accuracy bias	The closeness of a single measurement to the true value [11,12] Difference between the mean of measurements and the true value [11,12]
Precision	Closeness of agreement among replicate measurements on the same sample [11,12]
Reproducibility	Variation for the same measurement process among different instruments, operators and over a longer period of time [11]
Sensitivity Limit of detection	The smallest concentration of a analyte that can be reliably measured by an analytical procedure [13] Lowest analyte concentration likely to be reliably distinguished from the limit of blank[13]
Limit of blank	Highest 'apparent' analyte concentration found in a blank sample [13]

Results

HIV quantification assays: systematic literature search

The systematic literature search on the use of droplet digital PCR in the context of HIV quantification using patient samples resulted 19 articles that were included in this review (Figure 2).

HIV quantification assays can be divided into PCR- and non-PCR-based approaches and are focused on the different HIV forms/intermediates (Table 2). Across all reports, we observed that ddPCR was used for the quantification of multiple HIV-1 and HIV-2 forms/intermediates: total HIV DNA, 2-LTR circles, HIV unspliced-RNA and HIV multiple-spliced RNA (Figure 3). The majority of assays included HIV DNA-based applications (total HIV DNA and 2-LTR circles) and ddPCR was not only used for the quantification of integrated HIV DNA.

In the first part of this review, we have focused on reports that have compared ddPCR and qPCR platforms for different types of HIV assays.

In the second part, we have described further the applications of ddPCR in HIV research according to the type of HIV assay.

Comparison of ddPCR- and qPCR-based assays for HIV quantification

Since 2012, five reports have assessed and used ddPCR as a novel quantitative tool in HIV diagnostics and compared it to already-validated qPCR platforms. In this review, we have evaluated both platforms across these reports regarding their technical aspects (Table 3).

Assays for HIV DNA quantification

Henrich *et al.* performed the first comparison between ddPCR and qPCR for the quantification of total HIV DNA and 2-LTR circles [14]. This was conducted using serial dilutions of DNA standards on samples from HIV-infected patients (*n*=7) who were on or off antiretroviral therapy (ART). The results by qPCR and ddPCR quantification correlated well across all conditions and showed a similar type of detection sensitivity. However, differences in the absolute number of HIV DNA and 2-LTR circles were observed, with consistently higher numbers measured by qPCR. The authors suggested some potential explanations for this observation, including the effect of indirect quantification by standard curve for qPCR (overestimation), or decreased PCR efficiencies for ddPCR caused by incomplete DNA restriction or overloading of the droplets with target DNA copies (underestimation) [14]. Indeed, the latter was observed when loading more than 75,000 DNA copies per droplet, showing the need for samples to be diluted beyond this point. Finally, the authors have commented on the need for automated and reproducible threshold determination methods in order to increase the reproducibility of ddPCR quantification.
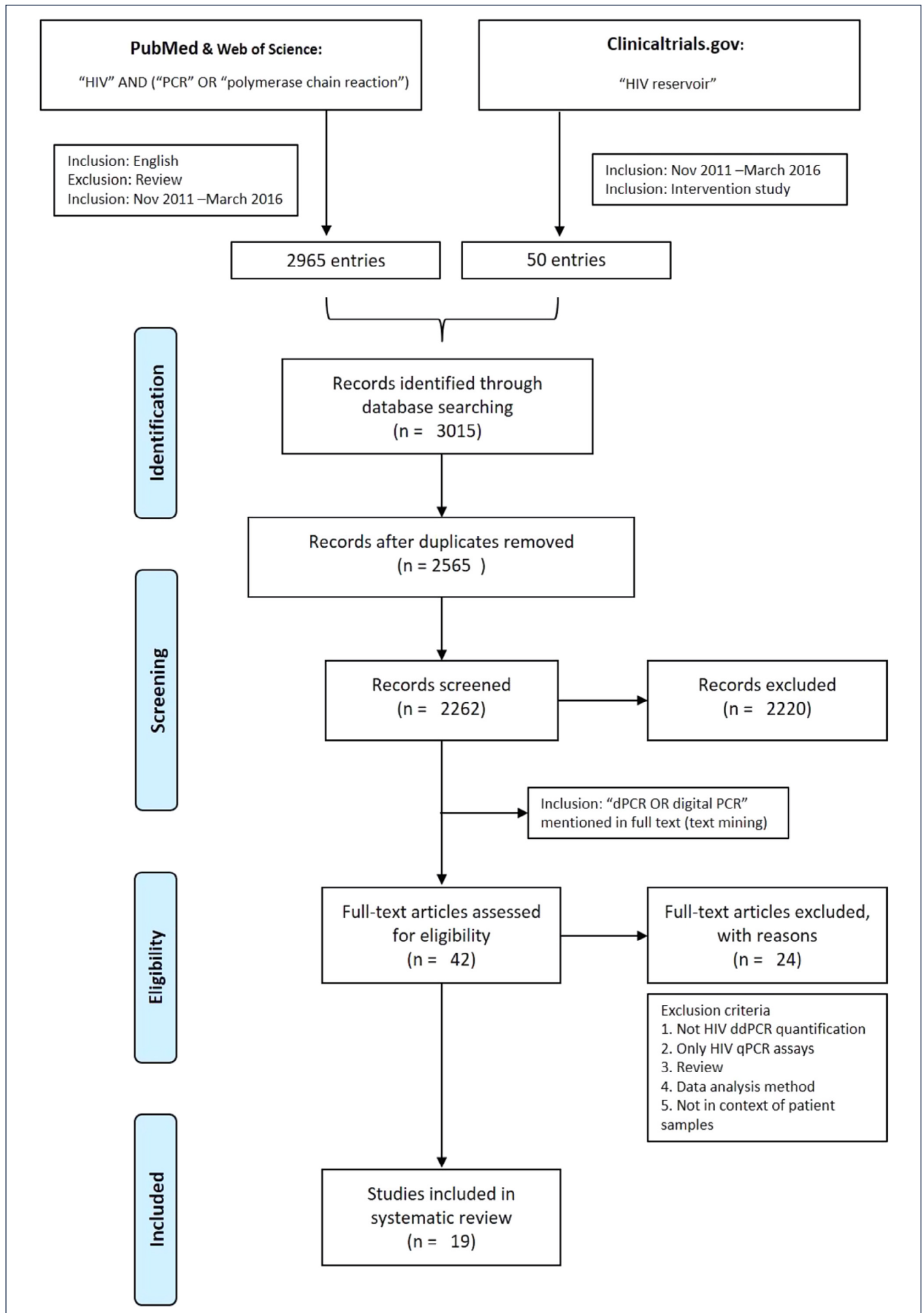
Figure 2. Overview of systematic pipeline used for this review

Table 2. Overview of reference methods for HIV reservoir quantification

HIV quantification method	What is measured
PCR-based methods	
Total HIV DNA	Total pool of HIV DNA in the cell
Integrated HIV DNA	Integrated fraction of HIV DNA in the genome
Episomal DNA	Non-integrated circular DNA forms
Unspliced RNA	Unprocessed RNA transcripts of HIV (full length HIV)
Multiple spliced RNA	Processed RNA HIV transcripts
Non PCR-based methods	
Viral outgrowth	The replication competent fraction of HIV viruses

A second comparison between ddPCR and qPCR for the quantification of total HIV DNA and 2-LTR circles was made by Strain *et al.*, which involved an analysis of over 300 clinical samples [16]. Here, ddPCR has shown an improved precision for total HIV DNA quantification and better accuracy for the quantification of 2-LTR circles. Furthermore, the authors reported that ddPCR offers an extra benefit for the quantification of micro-organisms with high sequence variability, such as HIV, because of an increased robustness to the mismatch of primers/probes with the target sequence. Indeed, in their experimental setup, mismatches were better tolerated by ddPCR than by qPCR, resulting in more accurate quantification results. Lastly, Strain *et al.* observed the existence of low numbers of false positive droplets in the negative template control (NTC) and presented a new data-driven threshold determination method to address the suboptimal threshold calculations made by ddPCR manufacturers' software (Quantasoft, Bio-Rad).

A third and final comparison for a total HIV DNA assay was conducted by Bosman *et al.* [28] and included two ddPCR platforms (QX100, Bio-Rad and Raindrop, Raindance) and a semi-nested qPCR on serial HIV DNA dilutions and samples from ART-suppressed patients [28]. Semi-nested qPCRs are specifically designed to reach higher sensitivity and accuracy as compared to the standard qPCR. The QX100 ddPCR showed equal sensitivity with the semi-nested qPCR but higher precision and efficiency, corroborating the findings by Strain *et al.* [16]. However, low numbers of false positive droplets in the NTCs were observed in the ddPCR. These droplets displayed high fluorescence, making them indistinguishable from the positive droplets in positive control samples. The semi-nested qPCR did not result in false positive samples. Hence, it was concluded that ddPCR outperforms qPCR for accurate quantification of low levels of HIV DNA, but that a semi-nested qPCR platform may be preferred if the focus is on discriminating between the presence or absence of HIV DNA.

Assays for HIV RNA quantification

Two reports have discussed the comparison of qPCR and ddPCR for HIV RNA assays, one for HIV-1 and one for HIV-2 [19,21]. Kiselinova *et al.* compared a semi-nested qPCR and ddPCR for the quantification of cell-associated HIV-1 RNA (unspliced and multiple spliced) [19]. This study highlighted that, in contrast to DNA quantification, RNA quantification by dPCR still requires a calibrator to correct for reverse transcription efficiency when transforming the template RNA to PCR-compatible cDNA. Furthermore, in this report, semi-nested qPCR showed better accuracy and sensitivity on a synthetic RNA standard, especially in the lower range of quantification. In contrast, ddPCR detected multiple spliced HIV-1 RNA in a larger portion of patients, resulting in higher sensitivity for that assay. However, a low number of false positive droplets in the NTC were observed in the ddPCR readouts

(as described elsewhere [16,28]). Interestingly, Kiselinova *et al.* [19] have also reported false positive droplets in NTCs in assays where either the forward or reverse primer was lacking, but with the other primer and probe in the reaction. This indicates that false positive droplets do not always arise from low levels of contamination, but may be artefacts from the specific type of assay and platform.

Concerning HIV-2 quantification, Ruelle *et al.* [21] presented an optimised ddPCR assay for plasma-free HIV-2 RNA (plasma viral load) measurement and found that ddPCR represented a more reproducible and sensitive assay as compared with qPCR. However, these authors have confirmed that false positive droplets in NTC remain an issue and underlined the need for an accurate threshold determination method before a transfer of ddPCR platforms into clinical testing.

ddPCR applications in HIV reservoir quantification studies

Droplet digital PCR has been increasingly used in different research settings to address specific HIV-related topics. Across all reports, ddPCR was most often used for the quantification of HIV DNA (total HIV DNA and 2-LTR) in the context of the latent HIV reservoir (Figure 3). In addition, different body compartments were targeted for absolute ddPCR HIV quantification, such as blood, rectal tissue and cerebrospinal fluid (CSF).

Total HIV DNA

de Oliveira *et al.* (2015) measured cell-associated HIV DNA levels by ddPCR in the CSF and paired peripheral blood mononuclear cell (PBMC) samples from 28 patients, 19 of whom were ART suppressed with an undetectable plasma HIV RNA viral load (<50 HIV copies/mL) [30]. They found that HIV DNA levels in CSF were comparable between ART-suppressed and non-suppressed patients, but lower in PBMCs of suppressed patients. The authors concluded that the CSF showed lower ART penetration and can harbour ongoing viral replication with replenishment of the viral reservoir.

King *et al.* performed ddPCR for HIV DNA detection in infants breast fed by HIV-infected mothers who were receiving post-natal antiretroviral prophylaxis [29]. Here, ddPCR HIV DNA quantification showed delayed detection of the virus with prophylaxis, resulting in missed diagnosis in infants when HIV testing was performed within 6 weeks after breastfeeding cessation.

In addition, a study performed by Yukl *et al.* [18] with blood and tissue samples from the so-called Berlin patient who was reported cured from HIV after receiving a bone marrow transplant from a homozygous CCR5Δ32 donor [32]. Here, ddPCR was used to characterise total HIV DNA in PBMCs in order to assess the impact of potential curative interventions by bone marrow transplantation [18]. No HIV DNA was detected in PBMCs but low levels of HIV DNA were detected by qPCR in rectum samples. The authors state that these low levels might represent a false positive result as no HIV sequences could be retrieved from rectum samples and HIV levels were near the limit of detection of the qPCR assay.

2-LTR circles

Hatano *et al.* monitored 2-LTR circles by means of ddPCR in a randomised control study in HIV-suppressed patients receiving either raltegravir intensification (*n*=15) or a placebo (*n*=16) [23]. They found an increase of 2-LTR circles over time in the group receiving raltegravir. The authors state that this might indicate a
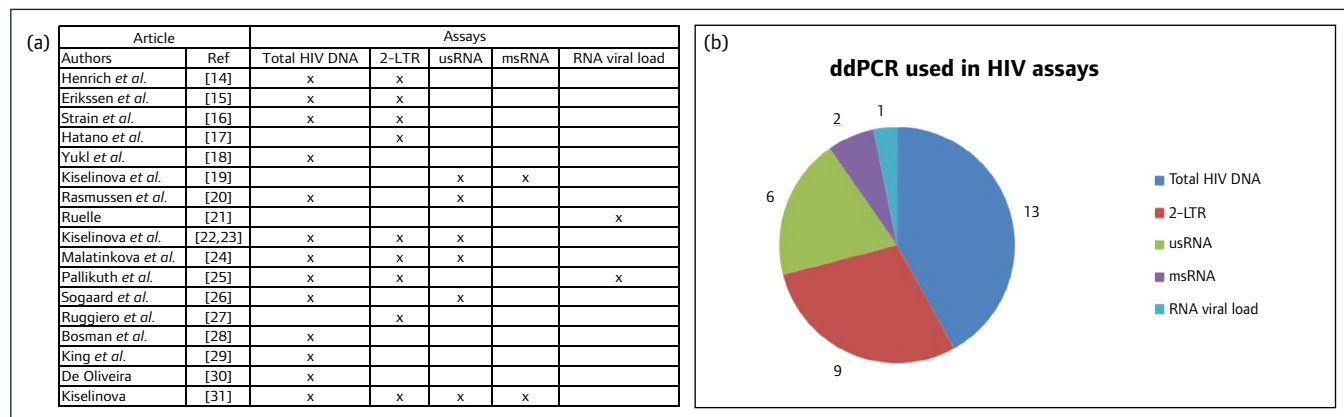
Figure 3. Overview of ddPCR HIV assays across all reports. Table view (a) and pie chart (b) of reported use of the assay across all reports based on Table 3

Table 3. Comparison of ddPCR and qPCR in HIV quantification assays. Colour scale indicates whether qPCR (blue) or ddPCR (green) was superior on a certain technical aspect or when both platforms showed equal performance (orange). –: no data available

	Report	Virus	Assay	Accuracy and bias	Precision	Sensitivity	Limit of detection	Reproducibility
1	Henrich *et al.* [14]	HIV-1	Total HIV DNA	–	–	equal	–	–
			2-LTR circles	–	–	equal	–	–
2	Strain *et al.* [16]	HIV-1	Total HIV DNA	ddpcr	ddpcr	–	ddpcr	ddpcr
			2-LTR circles	ddpcr	ddpcr	–	ddpcr	ddpcr
3	Kiselinova *et al.* [19]	HIV-1	usRNA	qpcr	–	qpcr	equal	–
			msRNA	qpcr	–	qpcr	ddpcr	–
4	Bosman *et al.* [28]	HIV-1	Total HIV DNA	–	ddpcr	equal	–	–
5	Ruelle *et al.* [21]	HIV-2	RNA (viral load)	–	–	ddpcr	ddpcr	ddpcr

low level of ongoing replication that can be blocked by raltegravir intensification in ART-suppressed patients.

Another study that has looked at ART-suppressed patients (*n*=50) was conducted by Ruggiero *et al.* [24]. Here, ddPCR was used in the determination of 2-LTR circles in addition to a range of other PCR-based HIV quantification assays and flow cytometry for immunological markers (CD4 plus one of CD26/CD38/CD69, CD8 plus either one of CD38/HLA-DR/DP/DQ). No correlation was found between 2-LTR circles with the HIV reservoir or the immunological markers studied.

Total HIV DNA and 2-LTR circles

Pallikuth *et al.* looked into HIV permissiveness and persistence in a subset of peripheral blood central memory T cells, namely peripheral T follicular helper cells (Tfh) [25]. Total HIV DNA and 2-LTR circles were measured in chronically infected patients before and after 48 weeks of raltegravir-based ART. Total HIV levels were found to remain stable but those of 2-LTR circles decreased over 48 weeks of treatment in Tfh cells.

Eriksson *et al.* used ddPCR to monitor total HIV DNA and 2-LTR circles and compared them using a non-PCR-based method that detects replication-competent HIV (i.e. the viral outgrowth assay) [15]. They found a discrepancy between the two methods, in so far as PCR-based methods recorded higher levels of HIV than those of replication-competent virus detected by the viral outgrowth assays (VOA). The authors state that this might be due to the substantial amount of replication-deficient HIV sequences found in HIV-infected patients that are picked up by PCR-based assays but not by the VOA [15]. In addition, more recent results indicate that the VOA underestimates the amount of replication-competent HIV DNA [33].

Total HIV DNA and cell-associated HIV RNA

Three studies have assessed ddPCR for total HIV DNA and cell-associated HIV RNA quantification in various patient cohorts. A first study by Kiselinova *et al.* used ddPCR in HIV RNA and DNA measurements for the comparison of different ART regimens: a standard ART backbone (two nucleoside reverse transcriptase inhibitors) combined with nevirapine or a protease inhibitor [22,23]. No difference in HIV RNA and DNA was found between the two treatment regimens. A second study by Malatinkova *et al.* determined HIV DNA and RNA levels with ddPCR in two body compartments (blood and rectal tissue) in different patient cohorts: early treated seroconverters, chronically treated patients, ART-naïve seroconverters and long-term non-progressors (LTNP) [24]. This study confirmed that early treatment is associated with a small viral reservoir and low levels of RNA transcription as compared to later treatment initiation. However, total HIV DNA levels were still higher than those of LTNPs [24]. In a third study, Kiselinova *et al.* have looked further into the discrepancy between the quantification results of the VOA and HIV DNA- and RNA PCR-based methods in samples of patients on ART [31]. Here, total HIV DNA levels were found to be higher than the replication-competent levels measured by the VOA, corroborating earlier findings by Eriksson *et al.* [15]. Additionally, the pool of total HIV DNA correlated well with the replication-competent reservoir measured by the VOA. Hence, the authors concluded that the amount of latent replication-competent HIV in patients receiving treatment can be predicted by measuring total HIV DNA levels using PCR-based methods [31]. Therefore, these methods can still be a useful tool in HIV eradication studies.

A last example of the use of ddPCR for the quantification of cell-associated HIV-1 RNA and DNA is in HIV cure trials aimed

at eliminating the HIV latent reservoir. In this context, several groups have reported the effect of various latency-reversing agents (e.g. panobinostat, romidepsin) on stimulating the latent reservoir [20,26].

Discussion

There has been renewed interest in the concept of digital PCR since the introduction of new commercial (droplet) digital PCR platforms [6,9]. The dPCR platforms have promised to be less labour-intensive with lower turnaround times, which should be attractive to many HIV research groups. Hence, comparison with established qPCR platforms for nucleic acid quantification is required.

The findings of this review confirm reports on ddPCR and qPCR comparisons within the field of cancer diagnostics (prostate cancer) and quantification of other viruses (e.g. cytomegalovirus), suggesting that ddPCR offers greater precision, improved reproducibility but with similar sensitivity to qPCR [1–3]. Furthermore, ddPCR benefits from direct quantification without the need for standard curves and offers a platform that displays higher robustness to primer and probe mismatches with the target sequence, making this a desired platform for HIV quantification as sequence heterogeneity is often observed. However, it must be noted that the difference between ddPCR and the more sensitive semi-nested qPCR is less clear [19,28]. None the less, the transition from qPCR to ddPCR could simplify and improve routine clinical/virological testing. However, before ddPCR can be fully accepted as an improved alternative to qPCR, two remaining issues have been reported that are discussed below.

The first involves ddPCR sensitivity and the presence of false positive droplets. Multiple reports have stated the existence of highly fluorescent droplets in negative template controls containing template-free genomic DNA, referred to as 'false positive droplets' [16,19,28]. This phenomenon seems inherent to ddPCR platforms (i.e. QX100 and QX200 Bio-Rad) and lowers the sensitivity of ddPCR quantification. This issue is especially problematic in the context of a low level of viral detection, as is the case for HIV quantification in patients receiving optimal ART with suppressed plasma viral load [14,16]. Therefore, extra quality-assurance steps are mandatory and crucial in order to identify and exclude false positive droplets and to achieve a higher sensitivity. In this context, new dPCR technologies that implement quality control for identifying false positive droplets could offer a (hardware) solution for this problem.

The second issue involves the standardised threshold determination. Ruelle *et al.* commented on the need for a standardised automated threshold setting, as the software methods provided by the current ddPCR platform manufacturers are not disclosed and often result in suboptimal quantification [21]. In this context, three alternative threshold determination methods are described to further improve reproducible data analysis: clustering methods [16,34], global manual threshold [35] and ddpcRquant [36].

Strain *et al.* were first to suggest a clustering-based method to identify positive and negative droplet populations by calculating two thresholds to delineate these two populations. Droplets that fall outside these intervals, referred to as 'rain', are discarded and excluded from further concentration calculations [16]. Jones *et al.* have discussed a similar approach, 'definetherain', which is based on k-nearest neighbour clustering to discriminate negative from positive droplet populations [34].

However, our group has shown that droplets considered as rain can contain genuine HIV sequences with mismatches in the primer

or probe binding region [36]. Hence, the elimination of droplets can bias quantitative outcomes, especially in the context of some viral or bacterial genomes where a high frequency in sequence variation is observed [16]. The global manual threshold method offers a single threshold determination that is calculated as six times the standard deviation of the negative droplet population and, in contrast to definetherain, it includes droplets with intermediate fluorescence (rain) [35]. Both the definetherain and global manual threshold data analysis methods assume a normal distribution of the droplet fluorescence. However, this assumption seldom holds true and can affect the correct allocation of thresholds or clusters [36]. Therefore, we have recently described an alternative statistical framework, ddpcRquant, to allocate thresholds based on an extreme value theory, which is independent of the underlying distribution of the total negative droplets. The ddpcRquant algorithm also accounts for shifts in baseline fluorescence between samples that can alter the correct droplet allocation (code and web tool interface available at www.ddpcrquant.ugent.be) [36].

Conclusion and future perspectives

There is growing interest in improving the sensitivity/detection limit of qPCR. In this context, dPCR platforms could represent an improved solution. However, the currently available platforms still struggle to fulfil these promises and the needs of the different end-users across multiple research domains. For the application in viral detection, ddPCR holds an inherent advantage over qPCR because of higher robustness to mismatches between the template and primers/probe. On the other hand, the reports of false positive droplets in NTCs remain a hurdle when attempting to reach higher sensitivity and, although new threshold determination methods offer a more reliable means of quantification, they are not applicable to all ddPCR experimental setups. These issues will need to be further addressed with costs to be further reduced in order for ddPCR to make its way into the clinically accredited setting.

Acknowledgements

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Appendix 1: Text mining approach

All articles resulting from the initial screening were searched for full-text availability through Endnote (X7) and this resulted in 2206 full-text pdfs. Next, Linux Bash commands were used for text mining:

```
sfind . –name "*.pdf" –size –12M –print0 | xargs –0 pdfgrep –I –m 1 'droplet digital PCR | ddPCR' 1> ddpcr_articles.txt 2> error.txt
```

Appendix 2: Table of articles reviewed

	Article		Included	Exclusion criteria				
				1. Not HIV ddPCR quantification assay	2. Only HIV qPCR	3. Review	4. Data analysis method	5. Not in context of patient samples
1	Henrich	2012	Yes					
2	De Spiegelaere	2013	No					
3	Eriksson	2013	Yes					
4	Hatano	2013	Yes					
5	Jangam	2013	No		x			
6	Kibirige	2013	No			x		
7	Massanella	2013	No	x				
8	Selck	2013	No	x				
9	Strain	2013	Yes					
10	Yukl	2013	Yes					
11	Bharuthram	2014	No	x				
12	Beliakova-Bethe	2014	No	x				
13	Kiselinova	2014a	Yes					
14	Kiselinova	2014b	Yes					
15	Jones	2014	No				x	
16	Malatinkova	2014	Yes					
17	Mitchell	2014	No		x			
18	Rasmussen	2014	Yes					
19	Ruelle	2014	Yes					
20	Bosman	2015	Yes					
21	de Oliveira	2015	Yes					
22	Janocko	2015	No		x			
23	King	2015	Yes					
24	Kiselinova	2015	Yes					
25	Li	2015	Yes					x
26	Malatinkova	2015a	Yes					
27	Malatinkova	2015b	Yes					
28	Mock	2015	No	x				
29	Pallikkuth	2015	Yes					
30	Perez-Santiago	2015	No	x				
31	Procopio	2015	No	x				
32	Rosadas	2015	No			x		
33	Ruggiero	2015	Yes					
34	Sogaard	2015	Yes					
35	Trypsteen	2015	No				x	
36	Hong	2016	No		x			
37	Massanella	2016	No			x		
38	Sedlak	2016	No					x
39	Valentini	2016	No	x				
40	Var	2016	No	x				
41	Whale	2016	No	x				
42	Kiselinova	2016	Yes					