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Original research

Adaptation of the intact proviral DNA assay to a nanowell-based digital PCR platform

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A B S T R A C T

Quantification of intact proviruses is a critical measurement in HIV cure studies both *in vitro* and *in vivo*. The widely adopted 'intact proviral DNA assay' (IPDA), designed to discriminate and quantify genetically intact HIV proviruses based on detection of two HIV sequence-specific targets, was originally validated using Bio-Rad's droplet digital PCR technology (ddPCR). Despite its advantages, ddPCR is limited in multiplexing capability (two-channel) and is both labor- and time intensive. To overcome some of these limitations, we utilized a nanowell-based digital PCR platform (dPCR, QIAcuity from Qiagen) which is a fully automated system that partitions samples into nanowells rather than droplets. In this study we adapted the IPDA assay to the QIAcuity platform and assessed its performance relative to ddPCR. The dPCR could differentiate between intact, 5' defective and 3' defective proviruses and was sensitive to single HIV copy input. We found the intra-assay and inter-assay variability was within acceptable ranges (with coefficient of variation at or below 10%). When comparing the performance of the IPDA in *ex vivo* $CD4^+$ T cells from people with HIV on antiretroviral therapy, there was a strong correlation in the quantification of intact ($rs = 0.93$; $p < 0.001$) and 3' defective proviruses ($rs = 0.96$; $p < 0.001$) with a significant but less strong correlation for 5' defective proviruses ($rs = 0.7$; $p = 0.04$). We demonstrate that the dPCR platform enables sensitive and accurate quantification of genetically intact and defective proviruses similar to the ddPCR system but with greater speed and efficiency. This flexible system can be further optimized in the future, to detect up to 5 targets, enabling a more precise detection of intact and potentially replication-competent proviruses.

1. Introduction

In people with HIV (PWH) on suppressive antiretroviral therapy (ART), a persistent reservoir of $CD4^+$ T-cells harboring HIV DNA (provirus) is thought to be the main barrier to an HIV cure. This reservoir is the source of rebounding virus when ART is ceased and therefore ART is currently required lifelong. The majority of persisting proviruses in cells are defective, featuring large internal deletions or hypermutation, and therefore are not replication competent and are unable to contribute to viral rebound.[1,2] Accurate and high throughput assays that can quantify intact DNA are needed.

The 'intact proviral DNA assay' (IPDA) was recently developed to quantify intact proviruses.[3] It is a duplex droplet digital PCR assay (ddPCR) which simultaneously amplifies two proviral targets, one within the packaging signal (*psi*) near the 5' end of the viral genome and another in the *rev* responsive element within *env* to estimate the level of genetically intact proviruses.[3] The IPDA provides a high-throughput method able to discriminate and quantify intact and defective proviruses requiring relatively small sample sizes (≤5 million $CD4^+$ T cells) and less time and resources than other assays used in the field.[3,4] As the technique has become widely adopted, other groups have reported an assay failure rate of up to 28% in clinical samples with the majority of assay failures due to sequence heterogeneity in the *env* target region which led to the design of a secondary *env* primer/probe and recovery of *env* detection in 9/9 participants.[5]

All IPDA assays to date have used ddPCR (BioRad) which benefits

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from absolute quantitation without the need for a standard curve. However, it features only two channels, limiting its multiplexing capability, and the workflow requires partitioning of samples into droplets which can be labor- and time intensive. In contrast, Qiagen's QIAcuity digital PCR platform (dPCR) is a fully automated system which partitions samples into nanowells rather than droplets. The self-contained instrument primes, thermocycles, and optically detects fluorescence of each sample with up to 5-plex capability, requiring minimal sample preparation, thereby reducing hands-on time and error. Here, we adapted the IPDA assay to the QIAcuity platform and assessed its performance relative to ddPCR.

2. Methods

2.1. Participants and ethics statement

Assay validation was performed using genomic DNA (gDNA) from $CD4^+$ T cells isolated from cryopreserved peripheral blood mononuclear cells (PBMCs) from 11 PWH on ART collected by leukapheresis at The Alfred Hospital in Melbourne, Australia (n = 8) and University of California San Francisco (UCSF), San Francisco, California (n = 3). Inclusion criteria were documented HIV infection, aged 18 years or older and receiving ART with plasma HIV RNA <50 copies/mL for at least 3 years. Use of samples was approved by the Human Research Ethics committees at the Alfred Hospital in Melbourne, the University of Melbourne, and the Institutional Review Board at UCSF.

2.2. Plasmid constructs and cell lines

Full-length, 5' deleted and 3' deleted HIV plasmids used in this study have been previously described.[6] Genomic DNA was isolated (AllPrep DNA/RNA Mini Kit, Qiagen, Hilden, Germany) from the latently infected cell lines, J-Lat 10.6, and ACH2 cells. The HIV copy number from the plasmids and cells lines was determined using total HIV DNA qPCR, as previously described.[7]

2.3. Intact proviral DNA assay (IPDA)

The ddPCR (QX200, BioRad) platform was used for IPDA analysis, as previously described.[3,6] The same primer and probe sequences were used for the QIAcuity: HIV-1 *psi* Forward Primer (CAGGACTCGGCTTGCTGAAG), HIV-1 *psi* Reverse Primer (GCACCCATCTCTCTCCTTCTAGC), HIV-1 *psi* Probe (FAM – TTTTGGCGTACTCACCAGT – MGB), HIV-1 *env* Forward Primer (AGTGGTGCAGAGAGAAAAAAGAGC), HIV-1 *env* Reverse Primer (GTCTGGCCTGTACCGTCAGC), HIV-1 *env* Probe (VIC – CCTTGGGTTCTTGGGA – MGB), HIV-1 Hypermutant *env* Probe (CCTTAGGTTCTTAGGAGC – MGB, Integrated DNA Technologies), *RPP30* Forward Primer (GATTTGGACCTGCGAGCG), *RPP30* Reverse Primer (GCGGCTGTCTCCACAAGT), *RPP30* Probe (VIC – CTGACCTGAAGGCTCT – MGB), *RPP30 Shear* Forward Primer (CCATTTGCTGCTCCTTGGG), *RPP30 Shear* Reverse Primer (CATGCAAAGGAGGAAGCCG), *RPP30 Shear* Probe (FAM – AAGGAGCAAGGTTCTATTGTAG – ZEN/Iowa Black FQ, Integrated DNA Technologies, Coralville, United States). We also used a previously published secondary *env* primer/probe set[5] with the Secondary *env* Forward Primer (ACTATGGGCGCAGCGTC), Secondary *env* Reverse Primer (CCCCAGACTGTGAGTTGCA) and Secondary *env* Probe (VIC – CTGGCCTGTACCGTCAG – MGB).

2.4. QIAcuity digital PCR

The IPDA assay was adapted to Qiagen's QIAcuity Four 5-plex digital PCR System (Qiagen). The reaction mixture was assembled as follows: 10 µL QIAcuity 4X Probe PCR Master Mix (Qiagen), 900 nM forward and reverse primer, 250 nM probe (except for *psi* and *RPP30 Shear* probes which were added at 500 nM), 2 Units XhoI restriction enzyme, PCR-grade water (ThermoFisher Scientific, Waltham, United States) and DNA template in a final volume of 40 µL. Reaction mixtures were prepared in standard 96-well PCR plates, mixed and transferred into QIAcuity 26k 24-well Nanoplates (Qiagen) for partitioning using the Qiagen Standard Priming Profile. dPCR cycling conditions comprised enzyme activation at 95 °C for 2 min followed by 45 cycles of denaturation at 95°C for 15 s and annealing/extension at 59 °C for 30 s. Partitions were imaged with 500 ms exposure time, and gain set to 6 for both target channels. Qiagen's QIAcuity Software Suite (version 2.1.7) was used to determine sample thresholds using positive, negative and no-template control wells (NTC). 2D plots were analyzed and double-positive as well as single-positive partitions in the *psi*, *env*, RPP30 and RPP30 Shear target channels were quantified using the Lasso tool as part of the QIAcuity Software Suite. DNA shearing was determined using valid RPP30 and RPP30 Shear partitions. The number of intact, 5' defective and 3' defective HIV copies was reported based on the number of cells screened in each sample well.

2.5. Inter- and intra-assay variability

To evaluate the intra- and inter-assay variability of the IPDA on the QIAcuity platform, genomic DNA from J-Lat 10.6 cells was titrated to an input concentration of approximately 140 intact HIV copies. The *psi* and *env* double-positive partitions were corrected for DNA shearing as determined by the number of RPP30 and RPP30 Shear double-positive and single-positive partitions. Small single use gDNA aliquots were used to minimize template variability between plates. A minimum of four technical replicates of the same template were analyzed across five or more assay plates to assess assay variability (HIV assays n = 5, RPP30 assay n = 6). The number of positive partitions in the *psi*, *env*, RPP30 and RPP30 Shear target channels were analyzed using the original assay[3], as well as the secondary *env* assay.[5] The mean, standard deviation of the replicate wells and percent coefficient of variability (% CV) were quantified for each plate and primer/probe set. Intra-assay variability was determined by analyzing 4 replicate wells across a plate and then averaging the % CV derived from each plate (n = 6 plates). Inter-assay variability was quantified from individual wells across n = 6 plates.

2.6. Comparison of dPCR to ddPCR

$CD4^+$ T cells from PBMCs from PWH on ART were isolated by negative selection (EasySep Human $CD4^+$ T Cell Isolation Kit, Stemcell Technologies), gDNA was isolated (AllPrep DNA/RNA Mini Kit, Qiagen) and quantified using the NanoDrop One C spectrophotometer (ThermoFisher Scientific). Genomic DNA was aliquoted across multiple smaller vials to avoid repeated freeze-thaw cycles. To compare dPCR and ddPCR, four technical replicates with 500 ng gDNA input (for IPDA) and 25 ng input (for RPP30) were assessed for each participant and analyzed using both the QIAcuity dPCR system and Bio-Rad ddPCR system respectively. Replicate wells were merged prior to the data analysis. PBMCs from uninfected donors and no-template controls were included as negative controls, The Jlat10.6 genomic DNA served as a positive control, and all controls were used to determine the gating thresholds for each target channel. The ddPCR was performed as previously described.[6]

2.7. Data and statistical analysis

For *ex vivo* data, comparisons between groups were made using a Wilcoxon Rank Sign Test. A Spearman's rank correlation was performed to assess the relationship between intact and defective proviral quantification between dPCR and ddPCR.

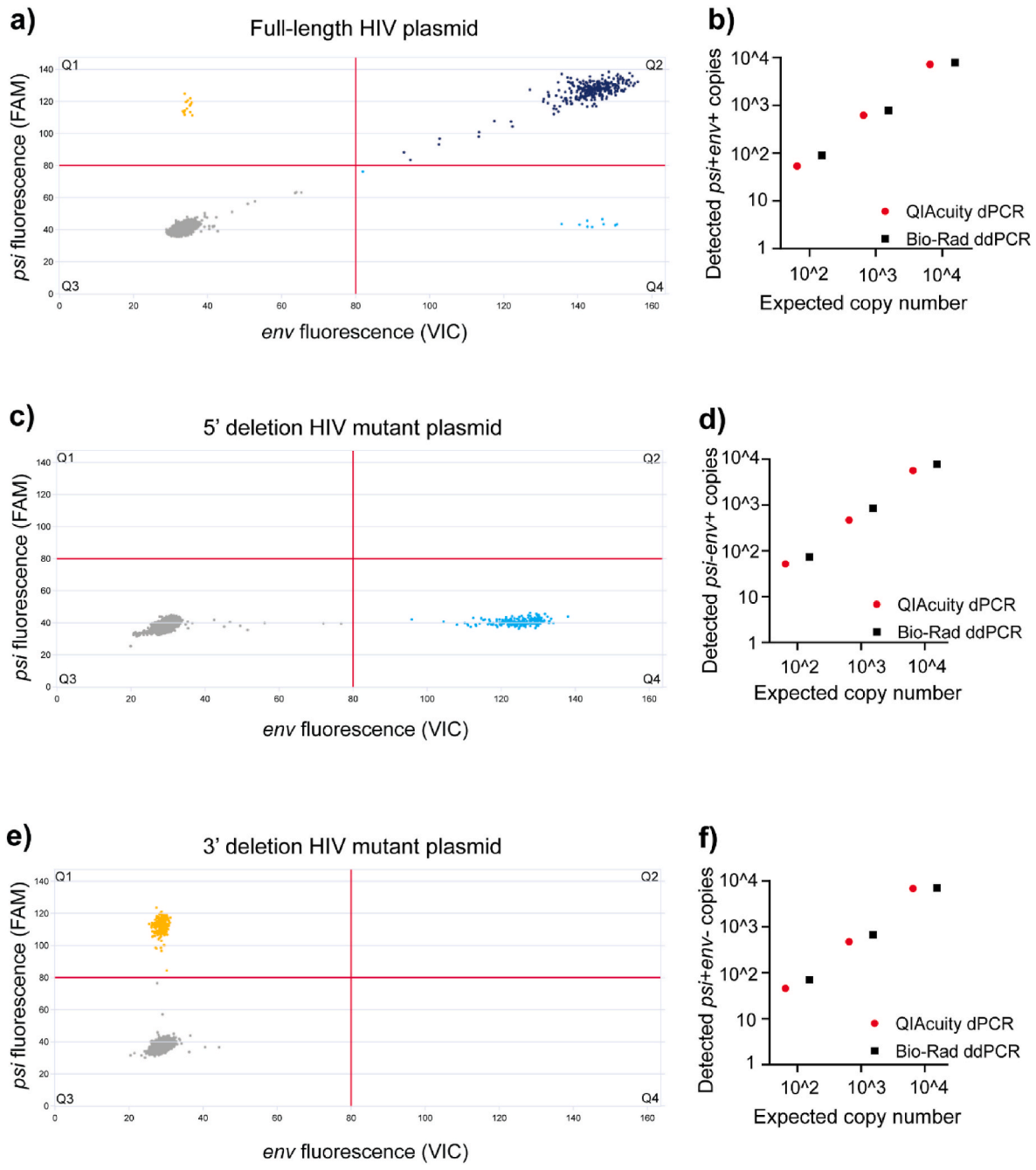

Fig. 1. Detection of defective and intact proviruses by dPCR and comparison to ddPCR. a) IPDA 2D QIAcuity dPCR plot using a full-length HIV plasmid. *Psi*-single-positive (Q1, yellow), *psi*- and *env*- double-positive (Q2, dark blue), double-negative (Q3, gray) and *env*-single-positive (Q4, light blue) partitions detected the full-length HIV plasmid with an estimated input copy number of 1000. b) Comparison of the QIAcuity dPCR and Bio-Rad ddPCR assays for detection of *psi*- and *env*-double positive copies with the full-length HIV plasmid with an HIV copy input of 10^2 to 10^4 copies. c) IPDA 2D QIAcuity dPCR plot using the 5' deletion HIV plasmid. Double-negative (Q3, gray) and *env*-single-positive (Q4, light blue) partitions were detected with the 5' deletion HIV mutant plasmid with an estimated 10^2 HIV copy input. d) Comparison of the QIAcuity dPCR and Bio-Rad ddPCR assays for detection of *env*- single positive copies with the 5'deletion HIV mutant plasmid with an HIV copy input of 10^2 to 10^4 copies. e) IPDA 2D QIAcuity dPCR plot using the 3' deletion HIV plasmid. *Psi*-single-positive (Q1, yellow) and double-negative (Q3, gray) partitions detected in the 3' deletion HIV mutant plasmid with an estimated 10^2 HIV copy input. f) Comparison of the QIAcuity dPCR and Bio-Rad ddPCR assays for detection of *psi*-single-positive copies in the 3'deletion HIV mutant plasmid with an HIV copy input of 10^2 to 10^4 copies. For b), d) and f), data points represent individual wells from one experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Validation of IPDA using dPCR

We first assessed the IPDA on the QIAcuity dPCR system with genomic DNA from the latently infected ACH2 cell line using parameters established for ddPCR.[3,5,6] We observed acceptable signal (>4-fold) to noise ratio (separation between the positive and negative partitions) for all assays after optimization. Positive signal for *env* and RPP30 VIC probes was detected at ~150 relative fluorescence units (RFU) in both assays (Suppl. Fig. 1 b and d). The detected fluorescence intensity of the FAM positive partitions in the *psi* and RPP30 Shear assays ranged around

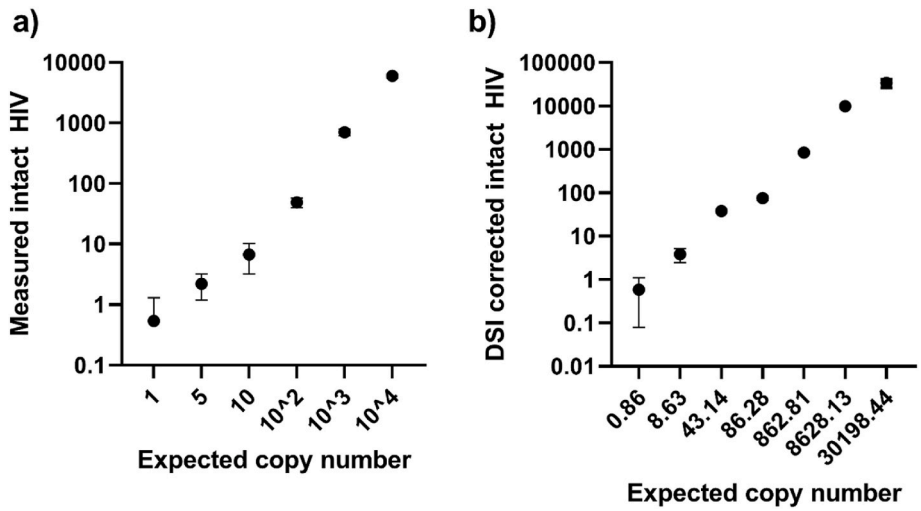

Fig. 2. Sensitivity of dPCR IPDA. The QIAcuity dPCR IPDA was used to quantify *psi*- and *env*-double-positive partitions and compared to the known input amount of HIV DNA using a) a titration of full-length HIV plasmid with a copy input of 10^4 to 1 copy and b) a titration of J-Lat 10.6 gDNA with a copy input of 3.5×10^3 to 0.1 copy with correction for DNA shearing. Data points represent the mean and standard deviation of n = 4 independent experiments.

60 to 80 RFU (Suppl. Fig. 1 a and c). As such, the FAM probe concentrations were increased from the published concentration of 0.25 μM–0.5 μM. Increasing the FAM probe concentrations to 0.5 μM increased the detected FAM signal to around 120 RFU in the *psi* assay and to around 80–100 RFU in the RPP30 Shear assay with minimal change in negative well fluorescence, therefore improving the signal to noise ratio of those assays. Increasing the concentrations of the FAM probes used in the *psi* and RPP30 Shear assays did not impact the detection of *env* and RPP30 in the VIC target channels, provided the thresholds were adjusted to avoid false *env*-positive partitions due to optical bleed-through from a high amplitude *psi* signal, when using conservative thresholding around the negative partitions.[5] The adapted IPDA assay protocol using the 0.5 μM FAM probe concentrations was then used for the validation of the IPDA on the QIAcuity.

To validate the specificity of the IPDA on the QIAcuity, the full-length HIV proviral plasmid pNL4-3 and variants with deletions in the 5' and 3' regions were used. Analysis of the 2D plots confirmed that the QIAcuity IPDA could differentiate between intact and defective proviruses, with detection of *psi*-single-positive (Q1, yellow), *psi*- and *env*-double-positive (Q2, dark blue), double-negative (Q3, gray) and *env*-single-positive (Q4, light blue) partitions with full-length HIV plasmid (Fig. 1a); detection of double-negative (Q3, gray) and *env*-single-positive (Q4, light blue) partitions detected with the 5' deletion HIV mutant plasmid (Fig. 1c); and detection of only p*si*-single-positive (Q1, yellow) and double-negative (Q3, gray) partitions detected with the 3' deletion HIV mutant plasmid (Fig. 1e). To compare dPCR and ddPCR assays, the full-length and mutant plasmids were quantified using an equal HIV copy number input of 10^2 to 10^4 copies. Analysis of *psi*- and *env*- double positive copies in the full-length HIV plasmid (Fig. 1b), *env*- single positive copies in the 5' deletion HIV mutant plasmid (Fig. 1d) and *psi*-single-positive copies in 3' deletion HIV mutant plasmid (Fig. 1f) confirmed similar copy number detection across both assays.

3.2. dPCR sensitivity

Next, the sensitivity of the dPCR IPDA was assessed by titration of full-length HIV plasmid and J-Lat 10.6 gDNA. A 10-fold serial dilution of HIV copy inputs from 10^4 copies to 1 copy was used for the full-length HIV plasmid and *psi*- and *env*- double-positive partitions were analyzed and reported as 'measured' intact HIV against the 'expected' copy number. The measured intact HIV and expected copy number were highly correlated down to a single HIV plasmid copy input demonstrating that the dPCR IPDA assay could accurately quantify intact proviruses to low-copy levels (Fig. 2a). Using the full-length HIV plasmid, we could reliably detect 10 copies of intact proviral DNA in 88% (14/16) of replicates tested, and 5 copies of intact proviral DNA in 63% (10/16) of replicates tested. The IPDA was linear over 4 orders of magnitude. Similar to the full-length HIV plasmid validation, 3×10^4 copies to 1 copy of HIV DNA in J-Lat10.6 gDNA served as template to analyze *psi*- and *env*- double-positive partitions. The number of *psi*- and *env*- double-positive copies was corrected for DNA shearing and reported as the DNA shearing index (DSI)-corrected intact HIV against the expected copy number (Fig. 2b; Supplementary Table 1). Using J-Lat10.6 DNA, we were able to reliably detect 5 intact HIV copies in all replicates tested (100%, 16 replicates), suggesting that dPCR can reliably detect <10 copies of intact proviral DNA. The median DSI was 0.39 (range: 0.38–0.41, n = 4) which is consistent with reports from other groups.[3,6] Taken together using either full-length HIV plasmid or gDNA from J-Lat 10.6 cells, the QIAcuity accurately detected intact proviruses to a level of 5–10 HIV copies.

Table 1
The intra-assay and inter-assay variability of dPCR.

Assay	%CV	
	Intra-assay	Inter-assay
psi	10.7	8.9
env	12.9	8.1
secondary *env*	9.8	5.7
RPP30	6.9	3.3
RPP30 Shear	6.6	4.2

3.3. Intra- and inter-assay variability of dPCR IPDA

To assess the variability of the IPDA within the QIAcuity nanoplate (intra-assay) and across different nanoplates (inter-assay), gDNA from J-Lat 10.6 cells with an intact HIV copy input of 140 copies was used as template. A minimum of four technical replicates were analyzed across at least five separate nanoplates to evaluate the assay variability of the original *psi* and *env* assays, as well as the secondary *env* and RPP30 and RPP30 *Shear* assays. Analysis of the percent coefficient of variability (% CV) for each assay revealed the intra- and inter-assay variability was within acceptable ranges for all five assays with % CV at or below 10% (Table 1), indicating high reproducibility within the QIAcuity dPCR system.

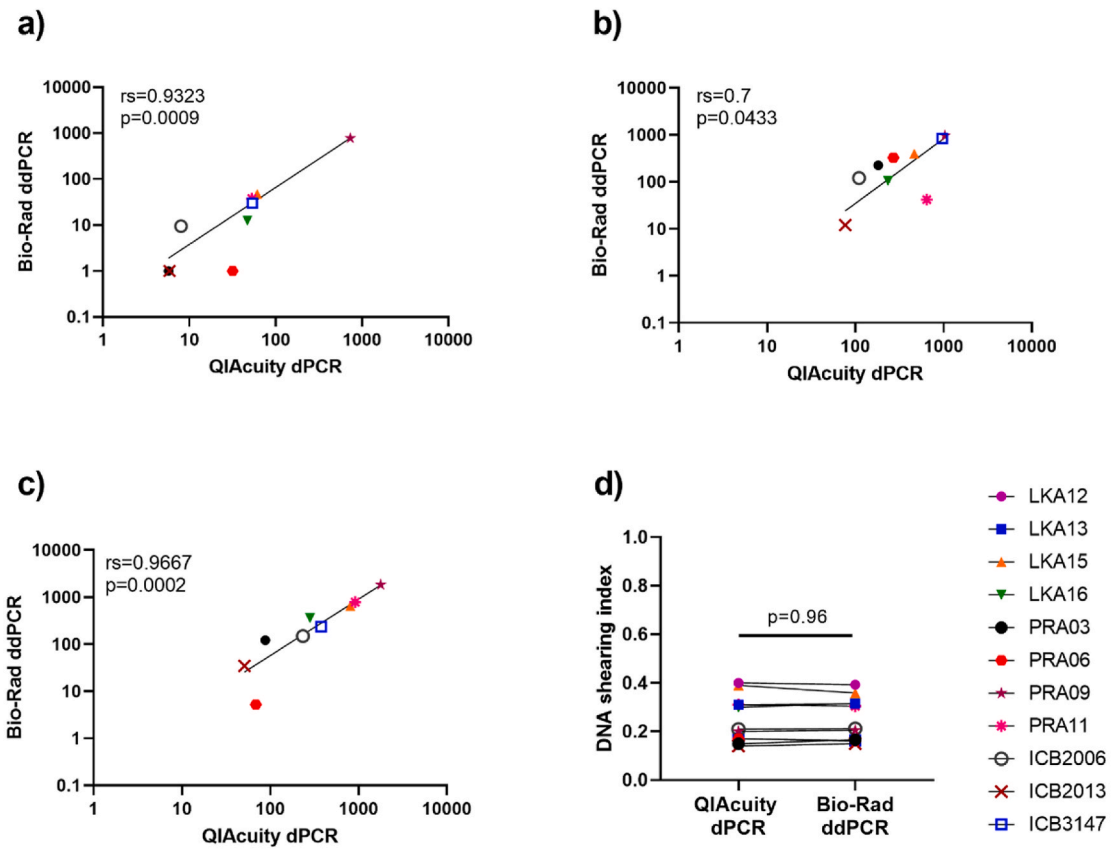

Fig. 3. Comparison of dPCR and ddPCR for detection of HIV DNA using CD4$^+$ T-cells from PWH on antiretroviral therapy (ART). Genomic DNA isolated from CD4$^+$ T cells from people with HIV (PWH) on ART (n = 11) was used to compare intact and defective HIV DNA using the QIAcuity dPCR and Bio-Rad ddPCR assay. Following DNA extraction from CD4+ T-cells, 500 ng DNA input in quadruplicate wells was assessed using the HIV assay and 25 ng DNA input for the RPP30 assay, to determine cell number and DNA shearing. We compared values derived from the dPCR and ddPCR for a) intact proviruses, b) 5' defective proviruses and c) 3' defective proviruses for 9/11 donors, using a Spearman correlation. d) DNA shearing (DSI) was compared for all 11 donors, using a Wilcoxon Rank Sign Test.

3.4. Comparison of dPCR to ddPCR IPDA in ex vivo CD4$^+$ T cells from PWH on ART

We next directly compared the dPCR and ddPCR assays[6] using gDNA isolated from CD4$^+$ T cells from PWH on ART (n = 11). A median of 2.95 × 10^5 (range: 1.49–4.51 × 10^5) cells were assayed on both dPCR and ddPCR instruments, and the number of intact, 5' defective and 3' defective proviruses was corrected for DNA shearing. *Env* amplification failure was detected in 2 of the 11 donors using both assays (Suppl. Fig. 2a) but could be recovered using alternate secondary *env* probes, as previously described[5] (Suppl. Fig. 2b). Those two donors were removed from the correlation analysis leaving a total of 9 donors (n = 9) for the comparison. We observed a strong correlation between dPCR and ddPCR assays in the quantification of intact (rs = 0.93; p < 0.001) (Fig. 3a), 5' defective (rs = 0.7; p = 0.04) (Fig. 3b) and 3' defective (rs = 0.97; p < 0.001) (Fig. 3c). The slightly lower correlation for the 5' defective provirus quantification was likely due to a discrepancy of the 5' defective provirus statistical ranking for one donor (shown as pink asterisk in Fig. 3b) causing a large effect on the correlation. Indeed, removal of this data point improved the correlation (rs = 0.92; p = 0.002). We were unable to find any differences in the fluorescent signals that would explain the discrepancy, and interestingly the quantification of intact proviruses was similar (52.96 intact copies/10^6 CD4 T cells for dPCR vs 37.82 copies/10^6 CD4 T cells for ddPCR). Further, although there were some variability in the number of proviruses measured in individuals patients (eg PRA11) by the two assays, overall we observed no statistical difference in the quantification of intact, 5' or 3' defective proviruses between the two assays (Supplementary Table 2; Supplementary Fig. 3). The DNA Shearing Index (DSI) for all 11 donors ranged between 0.14 and 0.4 with dPCR which was not statistically different to the ddPCR DSI of 0.15–0.39 (Fig. 3d; p = 0.96). Overall, using cells from PWH on ART, there was a high correlation between the Bio-Rad ddPCR and the QIAcuity dPCR assays for the quantification of intact and defective proviruses and no significant difference in the DSI.

4. Discussion

We demonstrate that the QIAcuity dPCR platform enables sensitive and accurate quantification of intact and defective proviruses similar to the established Bio-Rad ddPCR IPDA assay. The QIAcuity dPCR could detect down to 1–10 copies of HIV DNA, was linear over a dilution of 4 logs and had low intra- and inter-plate variability. Finally using CD4$^+$ T-cells from PWH on ART, the quantification of intact and defective proviruses and DSI by dPCR was consistent with previously published reports using cells from PWH on ART, predominantly infected with subtype B.[3,6,8]

The QIAcuity is a fully automated system which partitions genetic material into nanowells of a plate rather than droplets. The instrument then thermocycles and optically detects the fluorescence of each sample with up to 5-plex capability. This process requires minimal sample preparation thereby reducing hands-on time and error substantially. Of note, BioRad now has a fully automated platform (QX One) with four detection channels. The optional detection of up to 5 targets within sample partitions could ultimately provide a far more comprehensive analysis of intact and potentially replication-competent proviruses. Indeed this assay could be easily adapted to detect more than two targets

within the provirus, and could be applied to a cross-subtype IPDA (CS-IPDA)[9–11]; the triplex digital PCR[12]; and the quadruplex qPCR assay (Q4PCR).[13]

In conclusion, the QIAcuity dPCR system offers a versatile platform for the IPDA. The system has the added advantage of future higher order multiplexing that can detect more than two targets in one assay.

Author contributions

Conceptualization and study design: CT, MR, ST, SRL

Data acquisition and analysis: CT, CRC, YK, JO, AR, TAA, MJC, MR, ST, SRL

Manuscript preparation: CT, CRC, YK, JO, AR, TAA, MJC, MR, ST, SRL

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: SRL has received investigator-initiated grant funding from Gilead, Merck and ViiV Healthcare. She has been a paid member of advisory boards to Immunocore, Abivax, Abbvie and Gilead.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jve.2023.100335.

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