Low-Level Detection and Quantitation of Cellular HIV-1 DNA and 2-LTR Circles Using Droplet Digital PCR

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Abstract

Droplet digital PCR (ddPCR) is an emerging nucleic acid detection method that provides absolute quantitations of target sequences without relying on the use of standard curves. The ability of ddPCR to detect and quantitate total HIV-1 DNA and 2-LTR circles from a panel of patients on and off antiviral therapy was evaluated compared to established real-time (RT)-PCR methods. To calculate the dynamic range of ddPCR for HIV-1 DNA and 2-LTR circles, serial dilutions of DNA amplicons or episomes were determined by ddPCR as well as with RT-PCR. HIV-1 DNA from 3 viremic patients and 4 patients on suppressive antiretroviral therapy, and 2-LTR circles from 3 patients with low-level viremia was also quantitated. Copy numbers determined by ddPCR of serial dilutions of HIV-1 or human CCR5 DNA amplicon standards were comparable to nominal input copy number. The sensitivity of ddPCR to detect HIV-1 or CCR5 DNA was similar to that of RT-PCR. Low levels of 2-LTR circles were detected in samples from all 3 patients by both ddPCR and RT-PCR. ddPCR is a promising novel technology for the study of HIV-1 reservoirs and persistence, but further optimization of this novel technology would enhance the detection of very low-level viral genetic targets.

Keywords

droplet digital PCR; ddPCR; real-time PCR; HIV-1; HIV DNA; viral quantitation; 2-LTR circles

1. Introduction

Droplet digital PCR (ddPCR) is an emerging nucleic acid detection method that provides absolute quantitation of target sequences without relying on the use of standard curves. To perform ddCPR, the DNA target, fluorescently-labeled probe and the ingredients for a PCR reaction are partitioned into an emulsion of approximately 20,000 droplets, each of which ideally contains 1 or less copies of the target DNA. Using 96-well plates, 2 million PCR

reactions can be performed simultaneously (Hindson et al., 2011). Following PCR amplification, enumeration of both fluorescing and non-fluorescing droplets is performed, allowing the absolute quantitation of target molecules present in the original sample. To date, ddPCR has been validated primarily for measurement of germline copy number variation, detection and quantitation of rare alleles, and absolute quantitation of circulating fetal and maternal DNA (Hindson et al., 2011; Pinheiro et al., 2012).

The large-scale partitioning involved with ddPCR leads to greater precision and sensitivity for these applications when compared with real-time PCR (RT-PCR) (Hindson et al., 2011; Pinheiro et al., 2012). Partitioning reactions in picoliter wells may allow ddPCR to handle relatively large amounts of input DNA with little interference from PCR inhibitors. As result, ddPCR is a promising platform for investigation of very low-levels of viral genetic material, and there has been considerable interest in this technology for the sudy of HIV-1 reservoirs. Data are emerging that support ddPCR for the quantitation of low-levels of plasma HIV-1 RNA (Anderson et al., 2012), but the performance and applicability to the study of cell-associated HIV-1 DNA have yet to be fully described. Natural history studies of HIV-1 infection in the setting of antiretroviral therapy show that HIV-1 can persist indefinitely (Siliciano et al., 2003) because the viral genome integrates into host cell chromosomes. HIV-1 cDNA can also enter the cell nucleus, but fail to integrate, resulting in circularized molecules containing one or two copies of the long terminal repeat (LTR) region of HIV(Brussel, Delelis, and Sonigo, 2005; Butler, Johnson, and Bushman, 2002; Morlese et al., 2003). The development of novel methods to detect and quantitate low-levels of HIV-1 DNA is important to advance the study of viral persistence and eradication strategies. We evaluated the dynamic range of ddPCR and tested the ability of ddPCR to detect and quantitate HIV-1 DNA and 2-LTR circles from a panel of patients on and off antiviral therapy compared with traditional RT-PCR methods.

2. Methods

2.1. Patient samples, DNA extraction and preparation

Approval for the use of human material by the Brigham and Women's Hospital/Partners Healthcare Institutional Review Board was obtained prior to study initiation. In order to compare the performance of ddPCR and traditional RT-PCR for HIV-1 DNA, 5 million peripheral blood mononuclear cells (PBMCs) were obtained from 3 HIV-1-infected viremic patients and 4 patients on suppressive antiretroviral therapy. The PBMCs were washed in Hanks Balanced Salt Solution (HBSS) and pelleted. Following resuspension in 200 µL of phosphate-buffered saline, DNA was extracted using the QiaAMP Blood DNA Mini kit (Qiagen, Valencia, CA) as per the manufacturer's protocol. The DNA was eluted in 200 µL of elution buffer AE (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0), aliquoted and frozen at −80°C. Prior to PCR quantitation, the extracted genomic DNA was digested with MscI (New England Biolabs, Ipswitch, MA) because DNA strands longer than several hundred base-pairs do not package efficiently into the picoliter droplets. The MscI enzyme cuts at the 5′-TGGCCA-3″ sequence, which is absent from the proximal portion of HIV-1 *gag* and is thus unlikely to interfere with the PCR amplification of the HIV-1 DNA regions targeted in this study. A 50- µ l sample of the eluted DNA was digested in a total reaction volume of 60 µl at 37°C for 1 hour, followed by heat inactivation at 65°C for 20 minutes. Digested genomic DNA was then purified using the StrataClean resin (Agilent Technologies, Santa Clara, CA) as per the manufacturer's protocol.

To amplify 2-LTR circles, episomal DNA was extracted from 5 million PBMCs using the QIAprep Spin Miniprep Kit (Qiagen) following the instructions for the isolation of low-copy plasmids and eluted in 50 µL of nuclease-free water.

2.2. Real-time PCR quantitation of HIV-1 DNA and episomal 2-LTR circles

Following extraction, restriction enzyme digestion and resin purification, HIV-1 DNA was quantified using a validated Taqman real-time PCR method (Malnati et al., 2008). This assay allows for the precise quantitation of HIV-1 DNA down to 1 copy of an HIV-1 standard that comprises a conserved region in the LTR/gag common to nearly all group-M HIV-1 sequences (Malnati et al., 2008). Briefly, 10 µL of genomic DNA, 12.5 µL of Universal Taqman mastermix (ABI), 0.75 µL of 10 µM forward primer (5′-TACTGACGCTCTCGCACC), 0.75 µL of 10 µM reverse priemer (5″-TCTCGACGCAGGACTCG), and 1.0 µL of 5 µM FAM-MGB labeled probe (5′-FAM-CTCTCTCCTTCTAGCCTC) were added to each reaction well. HIV-1 standards were constructed by amplifying a cDNA region with primers that flank the region specified above from the HIV-1 reference strain HXB2 using the forward primer 5′-GGCTCACTATGCTGCCGCCC and the reverse primer 5′-TGACAAGCAGCGGCAGGACC. The exact number of DNA copies was quantified using a Nanodrop 2000c photospectrometer. Serial dilutions of the DNA standard ranging from 3 to 600,000 copies were run in addition to samples, no-template controls and genomic DNA from uninfected PBMCs. Amplifications were performed using the following PCR conditions: 95°C for 15 min; 42 cycles of 95°C for 15 s, 60°C for 1 min. HIV-1 DNA copy numbers were determined by comparing C_t values with those from the standard curves.

2-LTR circles were quantified by RT-PCR using a validated method (Butler, Johnson, and Bushman, 2002). Briefly, 10 µL of episomal PBMCs DNA were added to each PCR reaction in a master mix containing 25 µL of Taqman universal master mix, 11.5 µL of nuclease-free water, and 1.5 µL and 0.5 µL of 10 µM forward/reverse primer and FAM-MGB labeled probes, respectively(Butler, Johnson, and Bushman, 2002). In addition, a 149-bp sequence of the highly conserved human mitochondrial gene ND4 (Mishmar et al., 2003) was quantified from each sample using house primers MitoND4F 5′-ACCACTGACATGACTTTCCA and MitoND4R 5′-GTTAGGGGGGTCGGAGGAA, and FAM-MGB labeled probe 5′- CAACCACCCACAGCCTAATT. DNA standards and negative controls were run in parallel. The co-plasmid standard was constructed by ligating the 185-bp sequence HIV-1 LTR R-U5-U3 and the ND4 sequence as internal control using T4 DNA Ligase (Life Technologies, Grand Island, NY) followed by insertion into the pCR4-Topo TA plasmid (Life Technologies) following manufacturer protocol. The vector was amplified by transfection into Oneshot Top-10 electrocompetent cells (Life Technologies) followed by episomal DNA recovery by the Qiaprep Miniprep protocol (Qiagen).

2.3. ddPCR quantitation of HIV-1 DNA and 2-LTR circles

Five to 7.5 µL of MscI-digested, resin-purified genomic DNA was added to 10 µL of ddPCR 2× PCR master mix (Quantalife, Applied Biosystems), 0.75 µL each of 10 µM forward and reverse primers and 1.0 µL of 5 µM probe; the same primer and probes were used for real-time and ddPCR. Twenty microliters of the ddPCR master mix with genomic DNA was added to the droplet generator strips in addition to 60 µL of generator oil and placed in the automated droplet generator. The resulting picoliter droplet emulsion was transferred to a 96-well PCR plate taking care not to disrupt the droplets and the plates were sealed. PCR was then performed using the following program: 95°C for 10 min; 42 cycles of 94°C for 30 s, 60°C for 1 min; and 98°C for 15 min; the final high-temperature cycle cures the droplets. The plates were then placed in the ddPCR reader for enumeration of the number of positive and negative droplets based on fluorescence. The PCR probes have sequence specificity with the target DNA and fluoresce upon target amplification. The number of template molecules per microliter of starting material was then estimated by the

Quantalife ddPCR using an internal Poisson algorithm. Dilutions of the HIV-1 DNA standard were run in parallel with the genomic samples using the same protocol.

The 2-LTR circles and plasmid standards incorporating the 2-LTR and mitochondrial DNA sequences were quantified using similar master mix concentrations and reaction conditions and procedure above for genomic DNA with the exception of 40 PCR cycles prior to droplet enumeration. The probe and primer sequences were identical to those used in real-time PCR.

2.4 Statistical methods

GraphPad was used to generate standard curves from real-time PCR and ddPCR results and for comparison of HIV-1 DNA to sample standards and for cross-comparisons of ddPCR and real-time PCR standards. SPSS vs. 20 was used to calculate Pearson correlation coefficients and standard error measurements for comparison of patient sample quantitation.

3. Results

3.1. Real-time PCR and ddPCR quantitation of HIV-1 DNA

Copy numbers determined by ddPCR of serial dilutions of HIV-1 or CCR5 DNA standards are shown in Table 1. Linear regression correlation coefficients (R^2) for the log-transformed copy number between ddPCR results and expected copy numbers were all >0.98 and slopes were all > 0.92. Expected copy numbers measured by ddPCR were comparable to input standard values with the exception of the HIV-1 DNA amplicon standard; ddPCR detected approximately 60% fewer HIV-1 DNA copies than expected by the input standard copy number. However, the sensitivity of ddPCR to detect HIV-1 or CCR5 DNA was similar to that of RT-PCR; ddPCR and RT-PCR both obtained positive signals in 2 of 3 wells for the 3 copy standard. As expected, linearity of ddPCR decreased significantly when greater than 60,000 copies were added into each reaction well (data not shown). Of note, the ddPCR droplet generator led to inadequate droplet formation in approximately 1 out of every 16 wells, leading to loss of sample as noted in Table 1. An example of the visual readout of positive and negative individual ddPCR droplet reactions and the user-determined positive-negative threshold for HIV-1 DNA standard dilutions are shown in Figure 1.

Levels of HIV-1 DNA and the human CCR5 gene measured by RT-PCR and ddPCR for samples from 7 HIV-1 infected individuals (3 off ART with detectable viremia and 4 on suppressive ART) are shown in Table 2. HIV-1 DNA from MscI-digested samples from patients on and off ART ranged from **27** to 4894 copies per million PBMCs by real-time PCR whereas DNA copies by ddPCR ranged from **23 to 2289** per million PBMCs. Pearson correlation coefficients of sample HIV-1 and CCR5 DNA copy number by real-time and ddPCR were 0.98 and 0.85 respectively (all P<0.001). Overall, ddPCR enumerated approximately 10-60% fewer DNA copies compared with RT-PCR, but demonstrated similar detection sensitivity. On average, higher copy numbers of HIV-1 DNA were measured from subjects off ART by both quantitation methods.

3.2. Comparison of RT-PCR and ddPCR quantitation of HIV-1 2-LTR circles

Table 1 shows the correlations between copy numbers determined by ddPCR and the nominal copy number expected from 10-fold serial dilutions of 2-LTR circles and human ND4 mitochondrial co-plasmids. Human mitochondrial DNA levels were used to estimate the efficiency of DNA extraction and processing. Linear regression correlation coefficients (R^2) for log-transformed copy number were all >0.98 and slopes ranged from 0.95 to 0.99. The 2-LTR circles from the 0.75 copy co-plasmid standard was detected in 50% of wells by both ddPCR and RT- PCR.

2-LTR circles were quantified in PBMCs samples from 3 HIV-1 infected individuals, 2 from viremic controllers (viral load from 547 to 780 copies/mL) and one from a patient with persistent low-level viremia on ART (viral load = 203 copies/mL). Low levels of 2-LTR circles were detected in samples from all 3 patients by both ddPCR and RT-PCR (Table 3). However, there was considerable intra-sample variation in copy number by ddCPR for one sample. Given the relatively low volumes of eluted DNA, only two ddPCR wells could be tested per subject. Insufficient sample existed to perform the LTR assay on genomic DNA from patients included in analysis of total HIV-1 DNA.

4. Discussion

Digital droplet PCR is a promising novel technology for the detection and quantitation of viral genetic material. In this study, we demonstrate that the platform is generally comparable to traditional RT-PCR methods for both total HIV-DNA and episomal 2-LTR circles. Despite good correlations of both linear and episomal DNA standards between the two methods, differences in the number of genomic HIV-1 DNA copies and episomal 2-LTR circles were observed. Whereas ddPCR is a direct measure of target DNA concentration, real-time PCR relies on a standard curve to extrapolate the amount of target present. The PCR quantitation threshold cycle (C_t) of an unknown sample is compared to the C_t of known input/expected copy number of a dilutional standard. The copy number of the standard is calculated prior to PCR quantitation by measuring concentrations of a known linear or episomal DNA sequence with a known molecular mass. One potential cause of the discrepancy between the assays could be errors introduced by spectrophotometric determination of the DNA concentration, or subsequent serial dilution of the standards. It is also possible that PCR was less efficient under one set of PCR conditions, or that some of the genomic DNA remained undigested prior to droplet generation. Larger fragments may not have packaged efficiently into picoliter droplets, leading to an underestimation of HIV-1 DNA copies by ddPCR. By contrast, real-time PCR quantitation most likely would not be affected by size differences in genomic DNA strands as all genetic material is included in each reaction without packaging or partitioning.

Discrepancies between the two PCR approaches in measuring the number of 2-LTR circles may have been related to the fact that the input copy numbers were low overall. Despite relatively good linearity at low standard copy numbers for both real-time and ddPCR, a relatively small number of positive ddPCR droplets could dramatically alter the results in low copy-number samples. For example, 63 LTR circles/million PBMCs were quantified by ddPCR for one patient, but only 2.3 copies/million PBMCs by real-time PCR. There was, however, a high degree of variation between the observed copy numbers of the duplicate samples evaluated by ddPCR for this patient (3.7 and 123.6 copies). Furthermore, the maximum volume of input sample in ddPCR is 7.5 μL, whereas a larger sample volume can be included in real-time PCR. As a result, higher concentrations of DNA substrate may be required in ddPCR reaction wells in order to maximize sensitivity for detecting HIV-1 DNA at very low levels.

One inherent limitation of ddPCR as compared to RT-PCR is the need to dilute samples with more than 75,000 copies of the target DNA, as overloading the picoliter droplets results in a significant loss of linearity at higher copy numbers. While this problem has less impact on quantitation at the low copy numbers that would be expected for HIV-1 DNA (Richman et al., 2009; Siliciano et al., 2003), human chromosomal and mitochondrial DNA require significant dilution prior to assay.

Whereas ddPCR is a promising platform for the detection of total DNA, episomal DNA and viral RNA, the technology may not be compatible with certain methods used to detect

integrated HIV-1 DNA without involving standard curves. For example, *Alu*-PCR has become a standard technique to quantitate the integrated, proviral HIV-1 DNA fraction (Brussel, Delelis, and Sonigo, 2005; Liszewski, Yu, and O'Doherty, 2009). *Alu*-PCR relies on two PCR steps: the first amplifies the sequences between the nearest chromosomal *Alu* element and integrated HIV-1 DNA. However, because the target sequence is randomly inserted between *Alu* elements, different length strands from first-round PCR are created. The second step involves amplification and real-time PCR quantitation of only the HIV-1 sequence. However, this method requires a PCR standard generated by infection of a cell line containing randomly integrated HIV-1 DNA as input into the first-round PCR (Brussel, Delelis, and Sonigo, 2005; Liszewski, Yu, and O'Doherty, 2009). Although ddPCR could be used to quantitate HIV-1 DNA from the second PCR step, the need persists for an input standard.

Despite these limitations, ddPCR is a promising tool for the study of HIV-1 reservoirs and persistence. ddPCR and real-time PCR have similar dynamic ranges for linear and episomal DNA standards and ddPCR is generally comparable to real-time PCR for the detection and quantitation of HIV-1-DNA and 2-LTR circles. However, PCR inhibition during quantitation of large amounts of DNA may be reduced by the partitioning reactions into droplets, allowing for investigation of very low levels of HIV-1 without the use of repetitive sampling required by standard quantitative PCR methods. Employing statistical methods to define positive-negative cutoffs rather than relying on manual methods may also enhance ddPCR, and further optimization of this novel technology would improve the detection of very low-level viral genetic targets.

Acknowledgments

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Abbreviations

ddPCR	droplet digital PCR
RT-PCR	real-time PCR
PBMCs	peripheral blood mononuclear cells
LTR	long terminal repeat

Highlights

- ddPCR provides quantitation of sequences without the use of standard curves.

- The ability of ddPCR to detect HIV-1 DNA from patient samples was evaluated.

- The ability of ddPCR to detecting and quantitate HIV-1 DNA was similar to RT-PCR.

- ddPCR is a promising novel technology for the study of HIV-1 reservoirs.

- Further optimization would enhance the detection of low-level viral genetic targets.
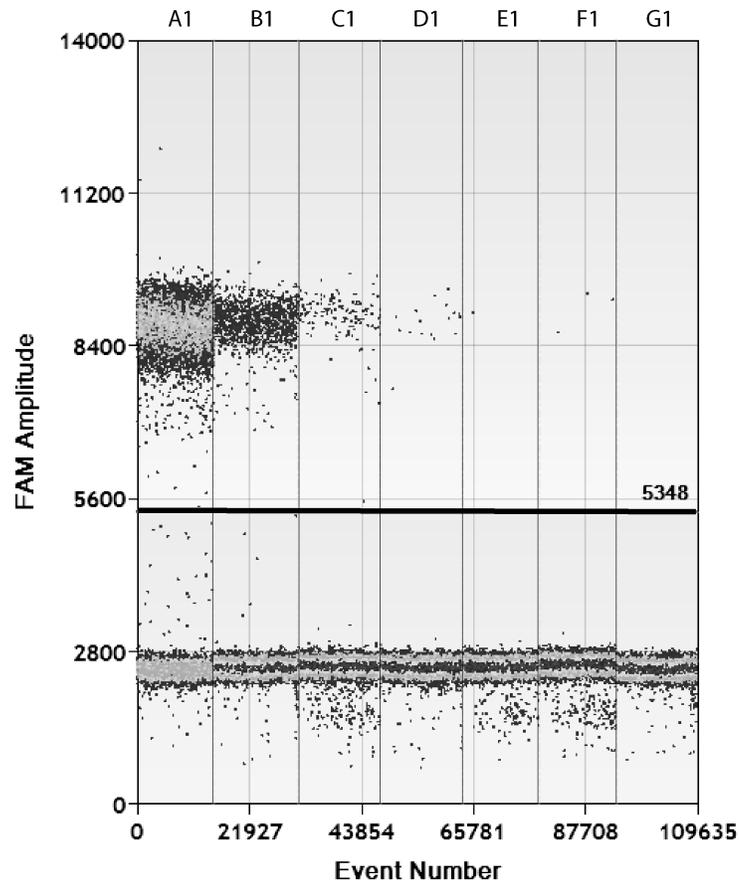
Figure 1.
Visual representation of ddPCR results for HIV-1 DNA standards after flow enumeration.
Positive droplets (upper cluster) and negative droplets (lower cluster) are shown for 6
serially diluted linear DNA standards (A1 through F1) and negative control (G1). The Y axis
represents fluorescent intensity and the X axis is the total number of events for each standard
dilution. The manually set threshold for droplet positivity is represented by the horizontal
line.

Table 1

ddPCR Detection and Quantitation of Serial Dilutions of HIV-1 DNA, Human CCR5, HIV-1 2-LTR and Human Mitochondria DNA Standards

Input Standard Copy Number per Reaction Well[a]	ddPCR (copies/reaction)	Standard Error	Wells Detected/Tested	% Level of Quantitation of Input Standard by ddCPR
HIV-1 DNA[b]				
60,000	37,706.7	1366.3	3/3	62.8
6,000	3,613.3	40.7	3/3	60.2
600	391.9	28.6	3/3	65.3
60	50.4	3.2	2/2[c]	84.0
6	3.3	-	1/2[c]	55.0
3	6.1	2.6	2/3	203.3
Human CCR5[b]				
60,000	68,200.0	200.0	2/2	113.7
6,000	6,740.0	180	2/2	112.3
600	742.0	54	2/2	123.7
60	70.6	0.6	2/2	117.7
HIV-1 2-LTR[b]				
75,000	71,100.0	1300.0	2/2	94.8
7,500	7,500.0	180.0	2/2	100.0
750	768.0	40.0	2/2	102.4
75	106.2	3.0	2/2	141.6
7.5	9.5	1.5	2/2	126.7
0.75	1.4	-	1/2	186.7
ND4 Mitochondria[b]				
75,000	64100.0	900.0	2/2	85.5
7,500	5760.0	-	1/1 [b]	76.8
750	752.0	36.0	2/2	100.3
75	85.2	7.2	2/2	113.6
7.5	8.7	6.1	2/2	116.0

Abbreviations: LTR, long terminal repeat

[a] Copy number based on serial dilutions of either linear or episomal DNA standard by DNA concentration and known molecular mass; input copy number = expected copy number for ddPCR quantitation.

[b] Linear regression coefficients (R^2) for the HIV-1 DNA, CCR5, 2-LTR and ND4 mitochondria standard curves were all >0.98 by real-time PCR [quantitation threshold (C_t) versus input copy number]

[c] ddPCR droplet generator failed to generate sufficient droplets for quantitation leading to fewer test wells

Table 2

Cell-Associated HIV-1 and Human CCR5 DNA from Clinical Samples Detected by ddPCR and RT-PCR

Sample	Plasma Viral Load	PBMC HIV-1 DNA (copies/10 µL ± SE)				CCR5 (copies/10 µL)		HIV-1 DNA per 10^6 PBMCs[a]	
		RT-PCR	WD/T[b]	ddPCR	WD/T[b]	RT-PCR	ddPCR	RT-PCR	ddPCR
Off ART									
A	13683	31 ± 1.0	3/3	15 ± 10.0	3/3	78116	91800	794	327
B	119612	269 ± 13.8	3/3	112 ± 34.8	3/3	115699	136800	4650	1637
C	7402	246 ± 18.7	3/3	133 ± 15.4	3/3	100536	116200	4894	2289
On ART									
D	<50	32 ± 5.0	3/3	22 ± 3.9	3/3	99917	99000	640	444
E	<50	26 ± 1.0	3/3	20 ± 5.7	3/3	69650	77600	747	515
F	<50	1 ± 0.4	2/3	1 ± 0.9	1/3	74842	86800	27	23
G	96	19 ± 1.8	3/3	17 ± 0.2	3/3	71479	84000	532	404

Abbreviations: RT, real-time; SE, standard error; PBMC, peripheral blood mononuclear cell

[a]Total HIV-1 DNA per PBMC number is a function of HIV-1 DNA copies and human genome equivalents (CCR5/2) detected in a set reaction volume (*e.g.* sample A: 31 HIV-1 DNA copies was quantitated in a reaction volume containing DNA from 39058 PBMCs = 794 copies/10^6 PBMCs).

[b]WD/T = number of wells detected/tested

Table 3

HIV-1 2-LTR Circle and Human Mitochondria Copy Number Detected by ddPCR and RT-PCR for Patients with Low-Level Viremia

Sample	Plasma Viral Load	HIV-1 2-LTR Circles (copies/10⁶ PBMCs ± SE)				ND4 Mitochondrial DNA (copies/10⁶ PBMCs) [a]	
		RT-PCR	WD/T [b]	ddPCR	WD/T [a]	RT-PCR	ddPCR
H (PLLV)	203	3.3 ± 0.8	2/3	3.3 ± 0.2	2/2	2.9×10^7	2.3×10^7
I (VC)	547	2.3 ± 2.2	2/3	63.7 ± 60	2/2	3.1×10^7	5.4×10^7
J (VC)	780	1.0	1/3	9.4	1/2	5.3×10^7	3.0×10^7

Abbreviations: PLLV, persistent low-level viremia on ART; VC, viremic controller; RT, real-time; SE, standard error

[a] Human mitochondria copy numbers differ between cell and patient and is therefore used to check efficiency of DNA extraction

[b] WD/T = number of wells detected/tested