SUPPLEMENTAL METHODS

Patient samples

HIV-1 infected peripheral blood mononuclear cells (PBMCs) were obtained from patients in follow-up at the AIDS Reference center of Ghent University Hospital (Belgium). Blood samples were drawn in 9 ml Venosafe® Plastic Tubes VF-109SDK (Terumo, Leuven, Belgium) after acquiring written informed consent. Samples were derived from two studies that were approved by the ethical committee of Ghent University Hospital (Reference number: B67020071646 (1) and Reference number: B670201111928). Both studies included 2-LTR quantification for comparison with clinical characteristics. Uninfected PBMCs were obtained from the blood of healthy donors. PBMCs were isolated following Ficoll density gradient centrifugation with Lymphoprep™ (Axis-Shield, Etten-Leur, Netherlands). Cells were manually counted using a hemocytometer counting grid, aliquoted in 1.5 ml tubes as dry pellets of 20 million PBMCs and stored at -80°C until further processing. In total 59 PBMCs samples were used in this study from patients on long-term cART with undetectable VL (n=44) and patients with detectable VL who either recently initiated cART (n=6) or were off therapy (n=9).

Generation of HIV-1 infected cells DNA

SupT1 cells were infected by spinoculation with a wild-type NL4.3 derived virus carrying *EGFP* gene in the Nef open reading frame (kindly provided by Dr. D.N. Levy, New York University college of Dentistry, New York, NY) (2). The cells were cultured for 14 days and infection was monitored by measuring intracellular p24 levels by flow cytometry. The p24 protein was stained

with a monoclonal mouse anti-p24 antibody (NIH AIDS-reagents AG3.0) from Dr. Jonathan Allen as previously described (3). Flow cytometry determined an infection rate of 16%. A serial 2-fold dilution series of SupT1 cells was made and each dilution was spiked in 20 million uninfected PBMCs.

Spiked normalization factor

To enable normalization to cell equivalents in the plasmid DNA isolates, a non-HIV plasmid (with a known copy number) was evenly spiked, as an internal reference, in the dry pelleted samples with the known number of cells before plasmid DNA isolation. The pSIF1-H1-Puro plasmid was used, a feline immunodeficiency virus derived lentiviral cloning and expression vector for shRNA templates (Supplemental Figure 1), (System Biosciences, Kampenhout, Belgium). Normalization to pSIF1-H1 is based on the assumption that the isolation efficiency of 2-LTR circles and pSIF1-H1 plasmid are similar (see Supplemental Results), since both are circular plasmids of similar length (pSIF1-H1: 6,261 bp). The plasmid was isolated from bacteria glycerol stock using QIAprep Spin Miniprep Kit (Qiagen, Venlo, The Netherlands) following manufacturer's protocol. In genomic DNA isolates, 2-LTR was commonly normalized to a chromosomal reference gene, here *RPP30*, where the number of cell equivalents was determined by calculating 2 copies of *RPP30* per cell (if diploid cell line used).

In the cell dilution series, the pSIF1-H1 was evenly spiked in samples for both DNA isolation methods, for subsequent comparison of circular plasmid DNA isolation efficiency between the two DNA isolation methods. The difference in pSIF1-H1 copy number between plasmid and genomic DNA isolates was corrected for different volumes of elution and the restriction mix for the genomic DNA isolates**.**

To assess the accuracy of 2-LTR quantification between the two methods in the dilution series, the linearity of measured and calculated 2-LTR copies was compared. To calculate the latter, the measured value of 2-LTR copy number per million cells in the first dilution point was taken as a starting copy number and 2-fold dilution was calculated.

Plasmid DNA isolation and genomic DNA isolation

Uninfected PBMCs with spiked SupT1 cells (dilution series) or PBMCs from HIV-1 infected patients were each divided in two equal aliquots of 10 million cells. One aliquot was used for genomic DNA isolation using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, The Netherlands). Total gDNA was eluted in 75 µl elution buffer, kept at 56°C for 10 minutes, to maximize DNA yield. The episomal DNA fraction was isolated from the second aliquot following the instructions for the isolation of low-copy plasmids with the QIAprep Spin Miniprep kit (Qiagen) as previously described (4, 5) and eluted in 25 µl elution buffer to maximize the DNA concentration.

An enzyme restriction digestion with EcoRI was performed on the genomic DNA (Promega, Leiden, Netherlands) with use of 17.3 µl gDNA in a total volume of 20 µl of restriction mix. This step is preferred for ddPCR as the fragmented DNA will be more uniformly distributed in all droplets compared to full length chromosomal DNA. The eluate of the plasmid DNA isolated fraction was used without prior restriction in downstream ddPCR amplification, because this eluate consists of small DNA fragments only. For each ddPCR well, 2 µl of the restricted gDNA sample or 2 µl plasmid DNA sample was used per 20 µl ddPCR mix (the 2-LTR, total HIV-1 DNA and pSIF assays). Total gDNA samples were 2-fold (patient samples) or 10-fold (cell line) diluted for the RPP30 assay to normalize to cell equivalents.

Primers and Probes

The primers and probes for 2-LTR, total HIV-1 DNA, *RPP30* and pSIF1-H1 quantification are summarized in Supplemental Table 1. 2-LTR assay is designed to span over the 2-LTR junction (5), total HIV-1 DNA copy number is determined with a primer-probe set designed on the *RU5* sequence of the HIV-1 genome (6). Spiked pSIF1-H1 was quantified by amplifying the Woodchuck hepatitis virus posttranscriptional regulatory element (*WPRE*) located in the vector. This assay was evaluated on ddPCR in samples containing pSIF1-H1 plasmid and to rule out false positive droplet calling by 2-LTR assay on the pSIF1-H1 sequence, the 2-LTR assay was run on pSIF1-H1 plasmid.

All primers and probes were purchased from IDT DNA Technologies (Integrated DNA Technologies, Coralville, Iowa), the probes were quenched with double quenchers, that is a 3′ Iowa Black dark quencher (IBFQ) in combination with an internal ZEN quencher.

Droplet digital PCR

DdPCR was performed with the QX100™ Droplet Digital™ PCR platform (Bio-Rad, Hercules, California). The ddPCR mix was made by adding 2 µl of sample to 10 µl 2x ddPCR™ supermix for probes (Bio-Rad), 500 nM of primers and 250 nM of probe in a final volume of 20 µl. The mix was placed in the 8 channel cartridge, 55 µl of droplet generating oil (Bio-Rad) was added and droplets were generated in the QX100™ droplet generator (Bio-Rad). Droplet in oil suspensions were transferred to an Eppendorf® 96 well plate (Eppendorf, Hamburg, Germany) and PCR was performed in the T100™ Thermal Cycler (Bio-Rad). DdPCR amplification reactions consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec denaturation and assay specific annealing/elongation temperature (Supplemental Table

1) for 60 sec. The droplets were read by the QX100™ droplet reader (Bio-Rad) and the data was analyzed with the QuantaSoft™ analysis software (Bio-Rad).

All samples were measured in triplicates for 2-LTR and total HIV-1 DNA and in duplicate for RPP30 and pSIF assays. For the 2-LTR assay, 104 NTCs with water were included for all ddPCRs and were used to assess the possible false positive events.

Normalization of the plasmid DNA isolated samples was performed by assessing the amount of pSIF1-H1 recovered by plasmid DNA isolation. Normalization to the amount of 2-LTR circles per 10 million PBMCs was then performed by taking into account the amount of pSIF1-H1 that was spiked per 10 million PBMCs as measured by initial cell counting. In genomic DNA isolates, normalization was performed by quantification of the internal *RPP30* gene of which two copies are present per cell equivalent.

Inhibition of ddPCR

Inhibition by gDNA was tested for two ddPCR assays quantifying two different fractions of the HIV-1 reservoir, i.e. a 2-LTR and a total HIV-1 DNA assay. DdPCR was run in triplicate on 5 serial 2-fold dilutions of gDNA isolated from healthy donors PBMCs with an equal amount of spiked HIV-1 infected gDNA in each dilution point. The serial 2-fold gDNA dilution and spiked HIV-1 infected gDNA resulted in 123, 63, 33, 18.2 and 12.4 gDNA ng/µl ddPCR mix.

Inhibition of ddPCR was tested at different volumes of restriction digestion mix (5, 4, 3, 2 and 1 µl of sample input) per 20 µl ddPCR mix and the total HIV-1 DNA was measured in single replicates. The restriction digestion mix contained 2 µl Buffer H, 0.2 µl Acetylated Bovine

Serum Albumin (10 µg/µl), 0.5 µl of Restriction Enzyme (EcoRI: 10 u/µl) (Promega) and gDNA sample in a total volume of 20 µl.

Statistical analysis

Statistical analysis of the data was performed with the use of R (RStudio®, Inc.) and Sigma Plot 12.5 software. Linear regression was used to analyze the correlation in both DNA isolation methods in dilution series. Wilcoxon Signed Rank Test was performed to assess statistically significant higher number of positive droplets detected in plasmid DNA isolates compared to genomic DNA isolates. Median values are shown with interquartile range (IQR). Bland-Altman analysis was used to assess the quantitative agreement between the two methods of 2-LTR quantification in patient samples with detectable 2-LTR in both isolates (n=27) and in patient samples with 2-LTR above 10 copies per million PBMCs in both isolates (n=17). Fisher's exact test was used to compare the detectability of 2-LTR in patient samples between the two methods.

Supplemental Table 1: Summary of ddPCR primers and probe sets used for 2-LTR, total HIV-1 DNA, *RPP30* and pSIF1-H1 quantification.

Assay	Annealing temperature (°C)	Genomic region	Name	Use in ddPCR	Sequence	Amplicon length (bp)	Reference
2-LTR	60	2-LTR junction	2-LTR F	Sense	5'- CTAACTAGGGAACCCACTGCT-3'		
			2-LTR R	Antisense	5'- GTAGTTCTGCCAATCAGGGAAG-3'		
			2-LTR	Probe	5'- /56-FAM/AGC CTC AAT /ZEN/AAA GCT TGC CTT GAG TGC /3IABkFQ/ -3'	226	(5)
Total HIV-1 DNA	58	*RU5*	RU5 F	Sense	5'-TTAAGCCTCAATAAAGCTTGCC-3'		
			RU5 R	Antisense	5'- GTTCGGGCGCCACTGCTAGA-3'		
			RU5	Probe	5'-/56-FAM/CCAGAGTCA/ZEN/ CACAACAGACGGGCACA/3IABkFQ/-3'	130	(6)
RPP30	60	*RPP30*	RPP30 F	Sense	5'-AGATTTGGACCTGCGAGCG-3'		
			RPP30 R	Antisense	5'-GAGCGGCTGTCTCCACAAGT-3'		
			RPP30	Probe	5'- /56-FAM/TTCTGACCT/ZEN/GAAGGCTCTG CGCG/3IABkFQ/ -3'	64	
pSIF	60	*WPRE*	WPRE F	Sense	5'-CCGTTGTCAGGCAACGTG-3'		
			WPRE R	Antisense	5'-AGCTGACAGGTGGTGGCAAT-3'		
			WPRE	Probe	5'-FAM-TGCTGACGCAACCCCCACTGGT-TAMRA-3'	84	(7)

References

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