SUPPLEMENTAL RESULTS

In depth optimization of 2-LTR assay and normalization

For the modified plasmid DNA isolation, a reference plasmid (pSIF1-H1) was spiked in the isolates before DNA isolation and used as a normalization factor for 2-LTR quantification. The pSIF assay did not detect HIV-1 sequences, as was confirmed by undetectable results in the pSIF assay on a control sample containing the 2-LTR sequence. In addition, the ddPCR assay quantifying 2-LTR was negative when run on the pSIF1-H1 plasmid. This shows that the spiking of pSIF1-H1 plasmid and the quantification with the pSIF assay can be performed in samples without biasing the 2-LTR quantification.

To evaluate the use of pSIF1-H1 as a normalization factor, the plasmid DNA isolation efficiencies of both plasmids were compared *in vitro*. Plasmid DNA isolation was performed on HIV-1 infected SupT1 cells with spiked pSIF1-H1 and both plasmids were quantified by ddPCR, to know the starting copy numbers of plasmids. Subsequently, a second plasmid DNA isolation was performed on these samples and quantified by ddPCR to determine the recovery efficiencies of both plasmids. This comparison revealed an equal 2-LTR and pSIF1-H1 isolation efficiency (data not shown).

To compare the efficiency of plasmid DNA recovery in the two DNA isolation methods, a known amount of the reference plasmid was spiked in the dilution series of HIV-1 infected SupT1 cells, both for plasmid and genomic DNA isolation. In average 1.9-fold more pSIF1-H1

copies (total number of copies/sample) were recovered in plasmid DNA isolates compared to genomic DNA isolates.

To further evaluate the normalization strategy based on the spiking of the reference plasmid, the numbers of cell equivalents were compared by using the spiked pSIF1-H1 plasmid or an internal reference gene (*RPP30*) in genomic DNA isolates of the dilution series. The median number of cells per ddPCR well determined by pSIF1-H1 was 125,305.7 (IQR 112,392-145,594.4) and 141,500 (IQR 131,250-160,750) by *RPP30*. This data validate pSIF1-H1 spiking as an accurate normalization strategy for 2-LTR quantification by plasmid DNA isolation.

The average amount of cell equivalents per ddPCR well was higher after plasmid DNA isolation compared to genomic DNA isolation. In the dilution series, 6.1-fold more cell equivalents were quantified per ddPCR well in the plasmid DNA isolates (median=867,423.9; IQR 769,540.3-983,621.8) compared to genomic DNA isolates (median=141,500; IQR 131,250-160,750). In all HIV-1 infected patients tested (n=59), 12.7-fold more cell equivalents were quantified in the plasmid DNA isolates (median=610,256.4; IQR 155,641-1,243,589.7) compared to genomic DNA isolates (median=47,900; IQR 39,150-61,000).

Of note, in the 59 patient derived samples, a high variation in plasmid isolation efficiency was observed, by comparing the number of pSIF1-H1 copies/µl sample recovered by plasmid DNA isolation (median=258; IQR 62.8-485). This high variation in isolation efficiency shows that an internal reference should be used to correct for the variations in sample processing.

Since plasmid isolated DNA still contains a residual amount of chromosomal DNA, the remaining chromosomal DNA in the plasmid DNA isolates was assessed by *RPP30* and compared to the genomic DNA isolates in the dilution series. Plasmid isolated samples contained

8.4-fold less chromosomal DNA compared to the genomic DNA isolates. This indicates that up to 8-fold more cell equivalents can be used as input material for plasmid DNA isolation as compared to the maximum input for genomic DNA isolation without risk for ddPCR inhibition.

Taken together, these data show that a higher number of cell equivalents could be loaded in the ddPCR when using the plasmid DNA isolation method compared to the genomic DNA isolation method, because the load of chromosomal DNA is minimized by the isolation procedure. Furthermore, the enzyme restriction digestion is not required for plasmid DNA isolates, whereas for the genomic DNA isolates the restriction buffer further limits the amount of genomic DNA input in ddPCR. In addition, elution volumes can be further decreased by plasmid DNA isolation, providing an extra concentration of 2-LTR circles compared to genomic DNA isolation. This enhances the accuracy of the low abundant 2-LTR quantification method.

2-LTR circles quantification in 3 different patient groups

Between the three patient groups, a difference in 2-LTR copies per million PBMCs was observed. The highest number of 2-LTR copies per million PBMCs was measured in the group of patients who recently initiated cART and still had a detectable viral load (VL) (Supplemental Figure 3). Patients with detectable VL who recently initiated cART (n=6) had a median 2-LTR count of 29.9 (IQR 22.2-61.4) in plasmid and 32.3 (IQR 29.3-41.7) in genomic DNA isolates and patients off therapy (n=9) had a median 2-LTR count of 26.3 (IQR 9.4-45.3) in plasmid and 24.8 (IQR 11-39.6) in genomic DNA isolates. Patients on cART with undetectable VL (n=44) had a median 2-LTR count of 0.8 (IQR 0-2.4) in plasmid DNA and 0 (IQR 0-17.3) in genomic DNA isolates.

Fluorescence amplitude

In ddPCR a clear distinction between the population of positive and negative droplets is crucial for accurate quantification. This is especially true in samples with low abundant target templates, such as 2-LTR circles in patient derived samples. Therefore, the mean fluorescence intensity of the positive population and its difference to the negative droplets was compared between both DNA isolation methods. After excluding the undetectable samples, the mean fluorescence intensities of the positive droplets was significantly higher in the plasmid DNA isolates (median=1,954.7; IQR 1,228.7-2,877.6) compared to the genomic DNA isolates (median= 1,222.3; IQR 805.4-2,093.4), (p=<0.001); while the mean amplitude of negative droplets with both methods was similar (median= 312; IQR 295-318.7) and (median= 301.2, IQR 264.8-327.1) in the plasmid DNA isolates and genomic DNA isolates respectively. With higher fluorescence intensity of positive droplets, there is a better distinction of these events from the background, further enhancing the accuracy of the quantification technique (Supplemental Figures 4, 5).

Positive droplets comparison

To assess the differences in terms of amount of positive droplets per sample between both DNA isolation methods, Wilcoxon Signed Rank Test was performed on the raw positive droplet counts of the 27 detectable patient derived samples and of the dilution series. A significantly higher number of positive droplets was observed in plasmid DNA isolates, detecting 2.4-fold more positive droplets in plasmid DNA isolates compared to genomic DNA isolates (p=<0.001; Supplemental Figure 6).

Evaluation of ddPCR false positive events

Since ddPCR is known to produce a limited number of false positive droplets in some no-template controls (NTCs), a total of 104 NTCs were included in all 2-LTR assays performed for this study. 20.2% of the total NTCs (n=21) contained a single false positive droplet and 79.8% of the controls (n=83) were completely negative. None of NTCs resulted in more than 1 positive droplet.

The issue of false positive droplets and consequent limitation of ddPCR quantification especially of templates of low abundance was already addressed in a recent study (1) analyzing 42 NTCs for assays to quantify cell-associated HIV-1 RNA and several NTCs with ≤ 3 positive events were recorded. This was also reported by Strain et al., who found an average of between 0.1 and 0.4 false positive events per well (2). The issue of false positive events in ddPCR needs to be further investigated, especially for quantification of low abundant templates.

Assessment of ddPCR inhibition

The ability to increase the DNA input of ddPCR is crucial especially in quantification of targets of low abundance in patient samples, since the occurrence of inhibition limits the amount of gDNA that can be loaded. Hence, less 2-LTR templates are detected by assessing genomic DNA isolates. The data on ddPCR inhibition is supported by recently published data reporting inhibition of 2-LTR quantification when gDNA concentration surpasses 75 ng/µl gDNA and a lower number of droplets is formed at gDNA concentration higher than 150 ng/µl gDNA in ddPCR (2).

To assess the maximum input of gDNA that can be used in a ddPCR, the inhibition caused by gDNA load was tested. Inhibition was measured in triplicates of a serial dilution of total gDNA

(extracted from uninfected PBMCs) spiked with an equal amount of gDNA from HIV-1 infected cells. Inhibition of ddPCR was observed for both the total HIV-1 DNA and 2-LTR assays at gDNA loads above 63 ng gDNA/µl ddPCR mix which – assuming an equivalent of 150 cells per ng of DNA input – amounts to 189,000 cells in a ddPCR well (Supplemental Figure 7A, 7B).

Remarkably, at 123 ng gDNA/µl ddPCR (369,000 of cells in ddPCR well) a lower number of accepted droplets was generated compared to the other dilutions. The average numbers of accepted droplets in the first six dilutions were 13,713 and 12,185 droplets/ddPCR versus 9,992 and 7,827 droplets/ddPCR in the dilution of 123 ng gDNA/µl ddPCR for the 2-LTR assay and the total HIV-1 DNA assay respectively.

Restriction digestion is generally performed for ddPCR on chromosomal DNA to allow an equal distribution of gDNA in the droplets. Therefore, the restriction digestion buffer was assessed for its influence on ddPCR inhibition. A comparison of increasing volumes of restriction mix in the 20 µl ddPCR well (from 1-5 µl of restriction mix per 20 µl ddPCR mix) revealed inhibition in volumes higher than 2 µl using Buffer H for EcoRI restriction (Promega) (Supplemental Figure 7C). Similar data was observed for NEBbuffers 1, 2 and 4 (New England Biolabs, Leiden, Netherlands) and the MULTI-CORE™ buffer (Promega). NEBuffer 3 (New England Biolabs, Leiden, Netherlands) had the highest influence on inhibition and should be used with caution in ddPCR. These results show that an input of 100,000 – 200,000 cell equivalents of genomic DNA isolates in a restriction digest of maximally 2 µl is the maximum possible input in a 20 µl ddPCR mix for optimal ddPCR.

References

1. **Kiselinova M, Pasternak AO, De Spiegelaere W, Vogelaers D, Berkhout B, Vandekerckhove L.** 2014. Comparison of droplet digital PCR and seminested real-time PCR for quantification of cell-associated HIV-1 RNA. Plos One **9:**e85999.
2. **Strain MC, Lada SM, Luong T, Rought SE, Gianella S, Terry VH, Spina CA, Woelk CH, Richman DD.** 2013. Highly Precise Measurement of HIV DNA by Droplet Digital PCR. PLoS One **8:**e55943.