Accurate Quantification of Episomal HIV-1 Two-Long Terminal Repeat Circles by Use of Optimized DNA Isolation and Droplet Digital PCR

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Episomal HIV-1 two-long terminal repeat (2-LTR) circles are considered markers for ongoing viral replication. Two sample processing procedures were compared to accurately quantify 2-LTR in patients by using droplet digital PCR (ddPCR). Here, we show that plasmid isolation with a spiked non-HIV plasmid for normalization enables more accurate 2-LTR quantification than genomic DNA isolation.

With the persistence of a viral HIV-1 reservoir despite the use of combination antiretroviral therapy (cART) (1), there is a strong need for sensitive tools to characterize the viral reservoir and its dynamics. Recent data indicate that episomal unintegrated circular HIV-1 DNA (two-long terminal repeat [2-LTR] circles) serves as a marker for viral replication (2–4).

DNA isolation procedures for 2-LTR recovery were recently compared (5). Although 2-LTR circles were recovered more efficiently by total DNA isolation than by plasmid DNA isolation *in vitro*, the small number of patients ($n = 3$) assessed impeded an *in vivo* evaluation at the lower end of detection. This is especially important, because of the small abundance of 2-LTR circles in patients on cART (6, 7). PCR inhibition by excessive DNA load limits the input material per reaction of total genomic DNA (gDNA) (8), while plasmid DNA isolation which specifically isolates episomal DNA from the bulk chromosomal DNA allows input from larger amounts of cells.

Here, two sample processing procedures were compared to quantify 2-LTR episomal HIV-1 DNA by using droplet digital PCR (ddPCR) in peripheral blood mononuclear cells (PBMCs) from a large number of HIV-1-infected patients. This comparison was made by ddPCR, which provides direct absolute quantification with a sensitivity similar to or better than that of real-time quantitative PCR (qPCR), as shown in studies assessing low levels of plasma HIV-1 RNA (9), cell-associated HIV-1 RNA (10), total viral DNA (8, 11–13), and 2-LTR circles (8, 13). In addition, absolute quantification by ddPCR provides a direct measure to assess template loss in pre-PCR processing steps.

A modified plasmid DNA isolation method was optimized, using a reference plasmid (pSIF1-H1) (see Fig. S1 in the supplemental material), further referred to as pSIF, spiked in the samples before DNA isolation for normalization to cell equivalents (see Methods and Results in the supplemental material for in-depth optimization and Fig. S2 in the supplemental material for the workflow). The pSIF plasmid was quantified by detection of the Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) (see Methods and Table S1 in the supplemental material), further referred to as the pSIF assay.

Quantification of pSIF in plasmid DNA and *RPP30* in gDNA isolates revealed a higher number of cell equivalents per ddPCR

replicate in plasmid DNA than in genomic DNA isolates, i.e., 6.1-fold and 12.7-fold more cell equivalents in the dilution series and in the patient-derived samples ($n = 59$), respectively (see Results in the supplemental material). 2-LTR quantities were normalized to cell equivalents by using the pSIF reference plasmid in plasmid DNA isolates or to the chromosomal gene *RPP30* in genomic DNA isolates. Of note, a high variation in plasmid DNA isolation efficiency was observed by comparing absolute numbers of pSIF copies/μl by plasmid DNA isolation (median = 258, interquartile range [IQR] = 62.8 to 485). This high variation in isolation efficiency supports the necessity of using the spiked reference plasmid for normalization.

A standard 2-fold dilution series of *in vitro*-infected SupT1 cells was used to compare 2-LTR quantification in isolates obtained by both DNA isolation methods. A slightly higher variation between replicates was observed in genomic DNA isolates ($R^2 = 0.95$) than in plasmid DNA isolates ($R^2 = 0.99$), especially for the lowest dilutions (Fig. 1A and B).

To assess the applicability of the proposed assay in patient samples, 59 samples were collected from HIV-1-infected patients with undetectable viral loads on cART ($n = 44$) or with detectable viral
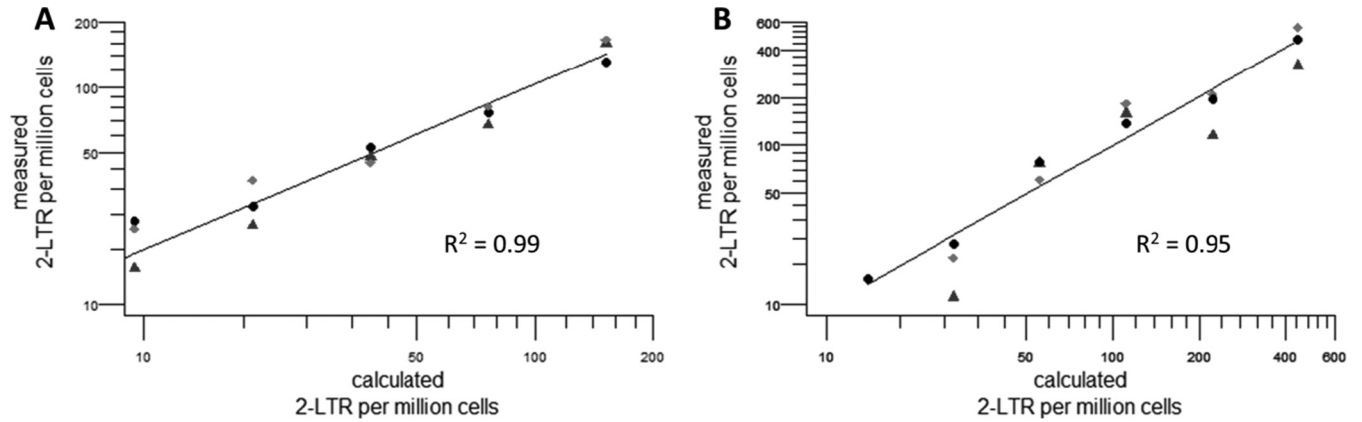
FIG 1 Quantification of 2-LTR circles in 2-fold dilution series of infected cells in both DNA isolation methods. (A and B) Linear correlations are observed with both dilution curves, but a higher variation within the three replicates was observed in genomic DNA isolates (B) than in plasmid DNA isolates (A).

loads who either recently initiated cART ($n = 6$) (median = 31 days, IQR = 5.5 to 125.3) or were off therapy ($n = 9$). The comparison of 2-LTR circles between the groups is shown in Results and Fig. S3 in the supplemental material. Samples were derived from two studies which were approved by the ethical committee of Ghent University Hospital (reference numbers B67020071646 [14] and B670201111928). Both studies included 2-LTR quantification for comparison with clinical characteristics.

Out of 59 HIV-1-infected patients, 27 (45.8%) patients had detectable 2-LTR counts with both methods. Bland-Altman analysis revealed an average agreement between both methods, with a mean difference (bias) ± standard deviation (SD) of 0.39 ± 1.27 $\log_{10}$ and with 95% limits of agreement (LoA) from −0.89 to 1.65 $\log_{10}$ (Fig. 2A). Since sampling error or infrequent false-positive calls may influence these data (8, 10), a more stringent comparison was made using only samples in which values of more than 10 2-LTR copies/million PBMCs were recorded with both DNA isolation methods ($n = 17$ samples). Bland-Altman analysis of these

samples revealed a mean difference (bias) of 5.86e−5 ± 0.44 $\log_{10}$, with 95% LoA between −0.44 and 0.44 $\log_{10}$ (Fig. 2B). 2-LTR copies were undetectable in 14 plasmid DNA samples and in 26 genomic DNA samples, indicating a higher sensitivity of the plasmid DNA isolation than of the genomic DNA isolation. This difference was significant by Fisher's exact test ($P = 0.03$), indicating a low probability that the difference is caused by random variability of false positives. Interestingly, the mean fluorescent amplitude of 2-LTR-positive droplets was higher in plasmid DNA isolates than in genomic DNA isolates (see Results and Fig. S4 and S5 in the supplemental material). This increases the sensitivity and the ease of discerning true-positive from false-positive events.

To compare the concentration of 2-LTR templates after DNA isolation in both methods, the amounts of positive droplets per sample were compared by Wilcoxon signed-rank test on the raw positive-droplet counts of the 27 detectable samples. A significantly higher number of positive droplets was observed in plasmid DNA isolates, with detection of 2.4-fold more positive droplets
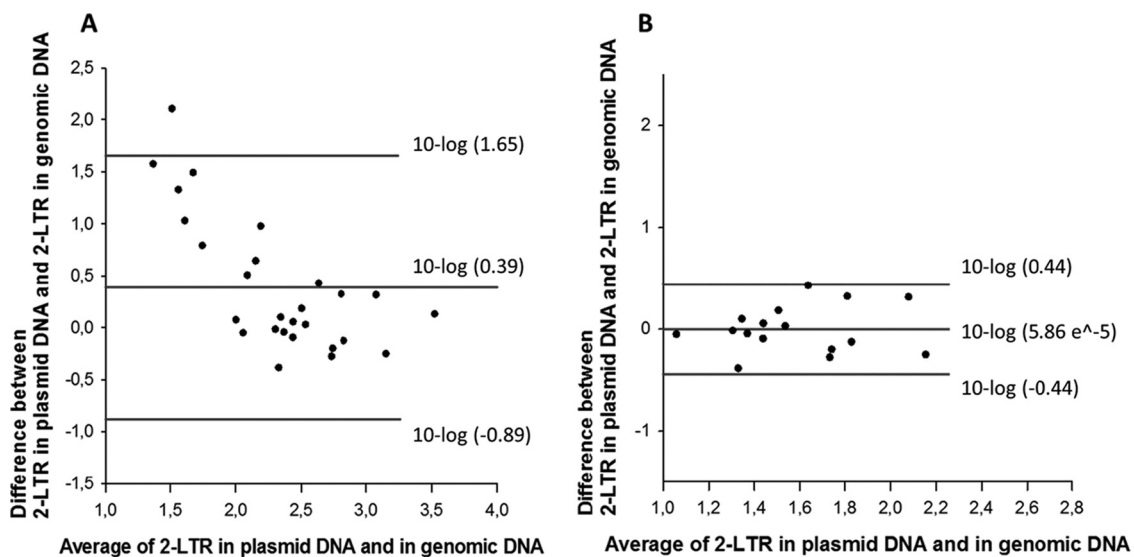

FIG 2 Agreement evaluation between the plasmid and genomic DNA isolation methods for the 2-LTR circle quantification in patient-derived samples. Bland-Altman plots comparing the 2-LTR quantification in both plasmid and genomic DNA isolates of 27 detectable samples (A) and of 17 samples with 2-LTR copies above 10 per million PBMCs (B). Mean differences and the 95% limits of agreement are shown on the graph.

than in genomic DNA isolates ($P = <0.001$) (see Results and Fig. S4 to S6 in the supplemental material).

The higher number of template molecules found in the plasmid DNA isolates can be explained by the higher concentrations of sample that can be loaded in ddPCR than by using genomic DNA isolates, which suffer from inhibition by bulk chromosomal DNA and the restriction buffer (see Results and Fig. S7 in the supplemental material). In the patient-derived samples, an average of 610,256 cell equivalents were loaded in the ddPCR well from the plasmid DNA isolates, whereas only 47,900 cell equivalents could be loaded for genomic DNA isolates (see Fig. S2 in the supplemental material).

In conclusion, these data show that plasmid DNA isolation combined with a reference plasmid for normalization provides an optimal method for 2-LTR circle quantification in patient-derived samples.

ACKNOWLEDGMENTS

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