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Protocols

Comparative analysis and generation of a robust HIV-1 DNA quantification assay

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ABSTRACT

HIV-1 infection cannot be cured due to the presence of the latent reservoir (LR). Novel cure or treatment strategies, such as “shock and kill” or therapeutic vaccination, aim to reduce or eradicate the LR. Cure strategies utilise robust DNA quantification assays to measure the change in the LR in low copy scenarios. No standard assay exists, which impedes the reliable comparison of results from different therapy and vaccine trials and HIV-1 total DNA quantification methods have not been previously compared. The HIV-1 long terminal repeat (LTR) has been shown to be the best target for DNA quantification. We have analysed two HIV-1 quantification assays, both able to differentiate between the variant HIV-1 DNA forms via the use of pre-amplification and primers targeting LTR. We identify a strong correlation ($r = 0.9759$, $P < 0.0001$) between assays which is conserved in low copy samples ($r = 0.8220$, $P < 0.0001$) indicating that these assays may be used interchangeably. The RvS assay performed significantly ($P = 0.0021$) better than the CV assay when quantifying HIV-1 total DNA in patient CD4+ T lymphocytes. Sequence analysis demonstrated that viral diversity can limit DNA quantification, however *in silico* analysis of the primers indicated that within the target region nucleotide miss-matches appear infrequently. Further *in silico* analysis using up to-date sequence information led to the improvement of primers and enabled us to establish a more broadly specific assay with significantly higher HIV-1 DNA quantification capacity in patient samples ($p = 0.0057$, $n = 17$).

1. Introduction

The development of antiretroviral therapy (ART) has been a major breakthrough in the treatment of human immunodeficiency virus type 1 (HIV-1) infection, effectively preventing the progression to acquired immunodeficiency syndrome (AIDS) (Brechtl et al., 2001). Despite this, ART cannot completely eradicate the virus due to the presence of a replication competent latent reservoir (LR) in different cell populations including long-lived resting CD4 + T cells that harbour pro-viral DNA integrated into the genome (Chun et al., 1997a; Finzi et al., 1997). Such infected cells can produce replication competent HIV-1, supporting rapid viral rebound following ART interruption (Davey et al., 1999; Joos et al., 2008; Rothenberger et al., 2015). Research is therefore focused on the development of novel approaches to reduce or eliminate the LR, with the aim of developing a functional cure for HIV-1 infection.

Therapeutic vaccination, administered during ART mediated virus suppression aims to stimulate the production of broad and effective immune responses, inducing sustained immune control of HIV-1 in the absence of therapy. A number of studies have explored the therapeutic potential of vaccination in both simian immunodeficiency virus (SIV) models (De Rose et al., 2008; Fuller et al., 2012, 2006; Hel et al., 2002, 2000; Lu et al., 2003) and in human trials (Barouch et al., 2013; Garcia et al., 2013; Lévy et al., 2014; Lu et al., 2004) with vaccine agents including DNA based vaccines expressing antigen, viral vectors expressing antigen, passive transfer immunotherapy, dendritic cells (DC) primed for HIV-1 antigen presentation or combinations of these (Mylvaganam et al., 2015). Generally, these studies have demonstrated that therapeutic vaccination can achieve reduced viral loads, increased time to viral rebound, reduction in size of the LR and in inducing stronger and more sustained immune response against HIV-1 (Mylvaganam et al., 2015). Alternatively, strategies which aim to completely eradicate the HIV-1 LR are popular in current research and

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Abbreviations: RvS, Rene van der Sluis assay; CV, Claire Vandergeeten assay; CRx, Christine Rouzioux

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clinical trials (Kim et al., 2018). These "shock and kill" approaches utilise latency reversing agents (LRAs) to induce activation of latently infected cells in the presence of ART, rendering those cells susceptible to cytolysis or immune clearance whilst limiting the chance of subsequent rounds of infection (Archin et al., 2017, 2012; Elliott et al., 2015; Margolis et al., 2016). Adding to this, recent approaches have explored the potential of a "lock in and apoptosis" strategy that when combined with the LRAs, utilises a novel compound to antagonise the viral gag protein and prevent virus budding whilst still inducing virus apoptosis (Tateishi et al., 2017).

Research focused on the reduction or elimination of the LR must utilise robust assays that can reliably and reproducibly measure the effect that the treatment or vaccine strategy has on the size of the LR. The quantification of HIV-1 DNA from peripheral blood mononuclear cells (PBMC) of patients via polymerase chain reaction (PCR) provides a useful tool to monitor the size of the viral reservoir and distinguish between different viral life-cycle stages. The initial assays were based around quantitative PCR measurements and adapted to be able to distinguish between single and 2-LTR circular forms (Kostrikis et al., 2002; Sharkey et al., 2000). These assays have subsequently been adapted, targeting different regions of the HIV-1 genome including gag, *pol* and the long terminal repeat (LTR) (Beloukas et al., 2009; Kabamba-Mukadi et al., 2005; Rouzioux and Avettand-Fenoël, 2018; van der Sluis et al., 2013; Vandergeeten et al., 2014; Yun et al., 2002). The strength of these assays is the rapid turn-around from sample collection to DNA quantification and the possibility to identify different HIV-1 DNA forms, such as integrated DNA, unintegrated linear DNA forms and 2-LTR circular DNA (De Spiegelaere et al., 2014; Kostrikis et al., 2002; Sharkey et al., 2000; van der Sluis et al., 2013; Vandergeeten et al., 2014). These different HIV-1 DNA forms have been used as markers of HIV-1 persistence in a number of different studies, reviewed here (Ruggiero et al., 2017). 2-LTR circular DNA is a product of abortive integration, and while some studies have suggested they are stable in CD4+ cells (Pace et al., 2013), they are considered markers of recent infection and ongoing replication notwithstanding therapy (Chun et al., 1997b; Koelsch et al., 2008; Murray et al., 2012; Sharkey et al., 2011; Zhu et al., 2011). Only assays targeting the viral LTR allow for the discrimination of different HIV-1 forms in addition to the fact that the LTR contains some of the most conserved regions of the viral genome (van der Sluis et al., 2013).

We have comprehensively analysed two HIV-1 DNA quantification assays, herein referred to as Vandergeetan, (CV) (Vandergeeten et al., 2014) and van der Sluis (RvS) (van der Sluis et al., 2013), both of which target highly conserved regions in the LTR region of the virus genome (van der Sluis et al., 2013; Vandergeeten et al., 2014) (Fig. 1). Both assays utilise a PCR pre-amplification step with primers designed to amplify all forms of HIV-1 DNA (total HIV-1 DNA) that have been fully reverse transcribed, including linear integrated, linear unintegrated and circular DNA forms, while excluding the short abortive transcripts. However, both assays are able to distinguish between total HIV-1 DNA and 2-LTR circular DNA with the use of alternative primer sets in the pre-amplification step. The CV assay is also able to distinguish integrated HIV-1 DNA via the use of primers targeting human *Alu* sequences, randomly dispersed in the human genome (Ruggiero et al., 2017; Vandergeeten et al., 2014). A prominent HIV-1 LTR based DNA assay, herein referred to as Rouzioux (CRx), was excluded from this comparison because this assay does not distinguish between different DNA types (Rouzioux et al., 2014). Furthermore, we have evaluated several calibration cell-lines, aiming to establish a stable and reproducible source of HIV-1 DNA for use as a standard curve. Additionally, we have analysed the primer sequences and used this information to establish an assay that would predict a broader specificity and increased sensitivity.

2. Materials and methods

2.1. Cell lines and calibration standards

HIV-1 quantification standards were produced from cell lines including 8E5 (CFAR 95), ACH-2 (CFAR 349) and J-Lat 10.6 (CFAR 9849), obtained from the NIH AIDS reagent program. Additionally, we utilised SupT1-14, a previously characterised cell line containing 14 HIV-1 copies per cell in comparing the assays (van der Sluis et al., 2013). Standards for the quantification of cell input were produced from dilutions of DNA derived from HEK293 T cells (ATCC CRL-3216). ACH-2, 8E5 and J-Lat 10.6 were maintained in RPMI-1640 medium (Fisher, 11875093) supplemented with 10% heat inactivated FBS (Sigma, non-US origin, F7524) and 1% pen-strep (Fisher, 15140122) at 37 °C with 5% CO_2. HEK293 T cells were maintained under the same conditions with advanced DMEM (Fisher, 12491015) used for culturing. Cells were passaged to a maximum of 10 cycles prior to DNA extraction using QIAamp DNA Blood Mini Kit, according to the manufacturer's instructions (Qiagen, 51104). DNA concentration and purity was assessed by Nanodrop analysis (Thermo Scientific, ND-200). The total number of cells and HIV-1 copy numbers were quantified using the CD3 and LTR quantification assays, respectively, and as previously described (van der Sluis et al., 2013; Vandergeeten et al., 2014). Standards were produced via a dilution series over a range of 5 logs. HIV-1 DNA standards were spiked with uninfected human genomic DNA to equalise DNA input in lower copy numbers.

2.2. Study population clinical sample preparation

The present study was approved by the Institutional Review Board of the Centre Hospitalier Universitaire Vaudois and all subjects provided written informed consent. CD4+ cells were isolated from PBMCs by negative selection using paramagnetic beads (StemCell Technologies) according to supplier's protocol. Purified CD4+ cells were digested via incubation with 0.1 mg/ml recombinant proteinase K (Roche, RPROTK-RO) in 10 nM Tris (pH 8.3) for 2 h at 56 °C. Lysate was centrifuged and supernatant recovered and used as input in the HIV-1 quantification assays. Proteinase K lysates were stored at −80 °C until use.

2.3. HIV-1 DNA quantification assays

Total HIV-1 DNA was quantified using both CV (Vandergeeten et al., 2014) and RvS (van der Sluis et al., 2013) LTR based HIV-1 DNA as well

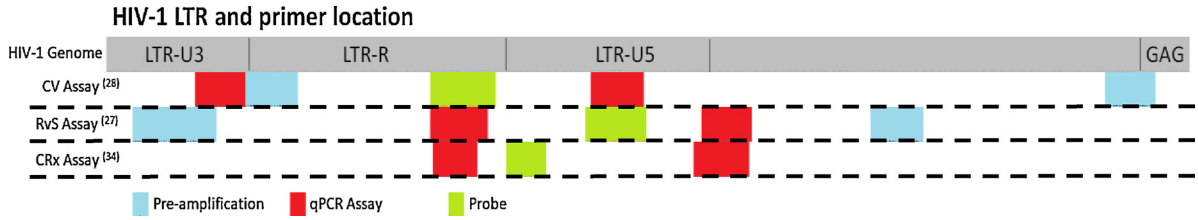

Fig. 1. HIV-1 LTR region: Locations of primers and probes for the CV, RvS and CRx assays. The numbers indicate the position on the HXB2 genome. For CV assay forward quantification primer anneals to Lambda T heel sequence on the forward pre-amplification primer.

as CD3 cell quantification assays. Primers and probes used in HIV-1 and CD3 DNA quantification are as described previously (van der Sluis et al., 2013; Vandergeeten et al., 2014). Additionally, a commercial cell quantification kit targeting the β-actin gene was used (ABI Applied Biosystems TaqMan β-actin Detection Reagent, 401,846). Pre-amplifications were performed in 25 μl reactions using Taq polymerase (Invitrogen, 10,342,020), as previously described (van der Sluis et al., 2013; Vandergeeten et al., 2014). Quantifications were performed in 20 μl reactions using Supermix-UDG (Invitrogen, 11,730,025) with the Qiagen Rotor Gene RotorQ, as described previously (van der Sluis et al., 2013; Vandergeeten et al., 2014). β-actin quantifications were performed according to the manufacturer's instructions. Reagent mixes for the quantification and pre-amplification PCR steps were adapted to the volumes used in this study, though the final concentrations remained the same as previously described (van der Sluis et al., 2013; Vandergeeten et al., 2014).

2.4. Sanger DNA sequencing

To sequence the primer and probe binding regions of both assays primers were designed in house to amplify the LTR region of patient samples (Table 1). Nested PCR was performed under the following conditions: 2 min (95 °C) followed by 35 cycles of 30 s (95 °C), 30 s (55 °C) and 1 min (72 ∘C) with a final elongation of 10 min (75 °C). The product of PCR 1 was diluted 1/10 in molecular grade water and this dilution was subsequently used as input for PCR 2. Amplification was analysed using gel electrophoresis and further purified using a Qiagen PCR Purification Kit (28104) prior to sequencing (GATC Biotech and Source Bioscience). Patient sequences were then aligned to primer and probe sequences using BioEdit software to identify mismatches. Following this, new primers were selected to exactly match the patient sample LTR region and used to quantify the total HIV-1 DNA using both assays, as described above (Table 1).

3. Results

3.1. Validation of assay quantification standards

Our aim was to examine the performance of the two assays CV and RvS and using the vast amount of sequence information available to date develop a new assay that will perform most optimally with the highest specificity and sensitivity. The incentive for this consideration was that both HIV-1 DNA quantification assays target the LTR of the HIV-1 genome, well established as the most conserved region of the genome, and furthermore both utilise a pre-amplification step allowing for the separate quantification of different viral life-cycle stages. In order to do so we initially aimed to define the cell quantification standard using a human genomic DNA input based on 293 T cells. We quantified the cell number using two methods; a commercial assay with primers targeting the human β-actin gene and a previously described assay targeting the human CD3 gene (Vandergeeten et al., 2014). We tested a 5 log standard range (10^5 to 10^1 cell equivalents) using both assays and found that they were within the optimum range of amplification efficiency (90–110%) and that there was no significant difference between either over 3 runs ($P = 0.8538$) (Fig. 2A and B). Based on this result we selected the CD3 quantification assay because it includes a pre-amplification step consistent with the HIV-1 DNA assays.

Further, we ran the two HIV-1 quantifications assays, RvS (van der Sluis et al., 2013) and CV (Vandergeeten et al., 2014), using 5-log serial dilutions (10^5 to 10^1 HIV-1 copies per input) of the J-Lat 10.6, 8E5, SupT-14 and ACH2 cell lines. We found no significant difference between qPCR efficiency of both assays over 6 runs ($P = 0.0552$). We next compared the HIV-1 DNA content in these cell lines, aiming to determine the most appropriate cell line for use as a quantification standard.

3.2. Evaluation of calibration cell lines

We evaluated HIV-1 integration model cell lines including ACH2, 8E5 and J-Lat as well as in 'in house' cell line, SupT-14, for their use as calibration standards. Cell lines were grown to 10 passages and the total HIV-1 per 10^6 cells was quantified following DNA extraction. Consistent with recent publications, we showed that HIV-1 copies per cell decreased in 8E5 cells from 1 to 0.2 copies (Fig. 3). Additionally, HIV-1 copies in ACH2 cells were found to increase from 1 to 4 copies per cell. On the contrary, HIV-1 DNA content was stable in both J-Lat 10.6 and SupT1-14, which contain 1 copy per cell consistent with recent studies (Sunshine et al., 2016) and 14 copies per cell as demonstrated previously (van der Sluis et al., 2013), respectively (Fig. 3). Based on these findings we used the J-Lat 10.6 to quantify patient samples in this study.

3.3. HIV-1 quantification in patient samples

We then compared the two HIV-1 DNA quantification assays using patient samples. Overall, a strong correlation was found between the results produced with the RvS and CV assays ($r = 0.9759$, $P < 0.0001$) (Fig. 4C). Nonetheless, the mean quantification of patient samples was significantly higher when using the RvS (3.385 Log10 HIV-1 copies/106 cells) HIV-1 assay compared to the CV assay (3.203 Log10 HIV-1 copies/106) ($P = 0.0021$) suggesting a slight advantage of RvS over CV when testing patient material (Fig. 4B). A possible explanation would

Table 1
Primers used for the amplification of patient sample LTRs and primers matched to patient sequences. For tailored and redesigned primers nucleotide positions that vary from the universal assay primer are underlined.

Name	Stage	Function	Sequence	Position on HXB2
Seqout-F	Sequencing PCR 1	Forward	CACACACAAGGCTACTTCCCTGATTAGCAGAACT	57–90
Seqout-R	Sequencing PCR1	Reverse	CTTAATACTGACGCTCTCGCACCCATCTCTCT	815–784
Seqin-F	Sequencing PCR2	Forward	GGGACTTTCCGCTGGGGACTTTCC	350–373
Seqin-R	Sequencing PCR2	Reverse	TCTCTCTCCTTCTAGCCTCCGCTAGTCA	790–763
RvS-preF_132	Pre-amplification	Forward	CAACCTTCAGAAGCTGCATAWAAGCAGCYGCT	409–440
RvS -preR_132	Pre-amplification	Reverse	AGCAAGCCGAGTCCTCGCGTC	688–707
RvS -preF_108	Pre-amplification	Forward	GAGCCCGTGGATGCTGCATAWAAGCAGCYGCT	409–440
RvS -preR_108	Pre-amplification	Reverse	AGCACAGCCGAGTCCTGCGTC	688–707
RvS –qF_ 124	qPCR	Forward	GGGCGCCACTGCTAGAGAA	625–643
CV-preF_124	Pre-amplification	Forward	ATGCCACGTAAGCGAAACTCTGGGTCTCTCTDGCTGGAC	452–471
CV -preR_124	Pre-amplification	Reverse	CCATCTCTCTCCCTTCTAGC	775–793
CV -preF_132	Pre-amplification	Forward	ATGCCACGTAAGCGAAACTCTGGGTCTCTCTDGCTAGAC	452–471
CV –preF_108	Pre-amplification	Forward	ATGCCACGTAAGCGAAACTCTGGGTCTCTCTDGTCAGA	452–471
RvS-preF-A	Pre-amplification	Forward	ARCCCTCAGAHGCTGCATAWAAGCAGCYGCT	410–440
RvS-preF-B	Pre-amplification	Forward	ARCCCTCAGAHGCTGCATAWAAGCAGCYGC	410–439

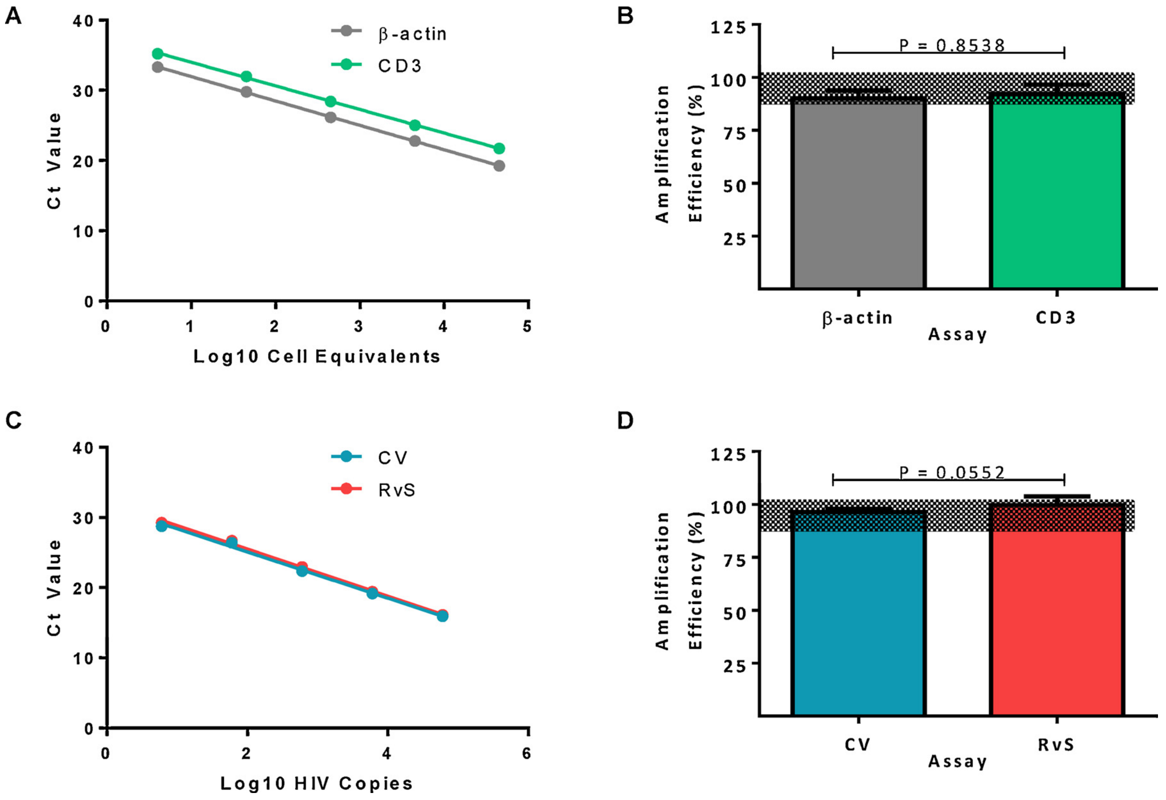

Fig. 2. Comparison of standards and assay performance: A) 5 log serial dilution of human genomic DNA quantified using CD3 and β-actin qPCR. B) Average amplification efficiency of CD3 and β-actin assays (n = 3). C) 5 log serial dilution of J-Lat clone 10.6 cells using CV and RvS. D) Average amplification efficiency of CV and RvS assays.

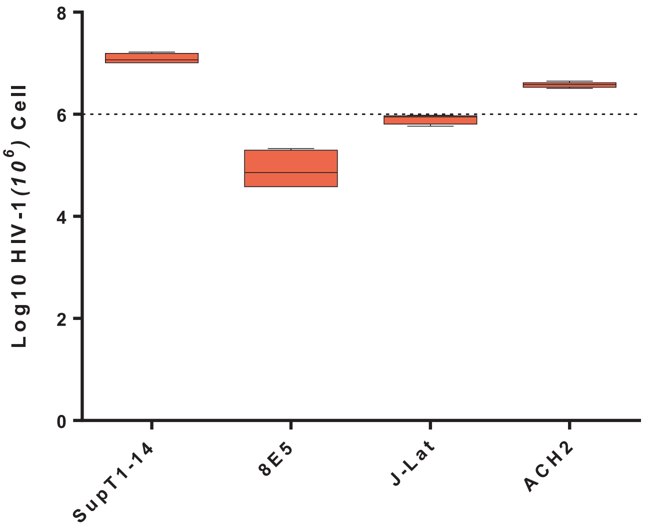

Fig. 3. Quantification using different cell lines: Quantification of three cell lines containing 1 HIV-1 copy per cell (8E5, J-Lat) and 14 HIV-1 copies per cell (SupT1-14). A five log dilution series for each cell line was performed and used as input for the assay. Quantification of each dilution was pooled and standardised to determine the average HIV-1 copies per cell.

that the CV amplified product is longer than the RvS thus affecting the amplification efficiency. Furthermore, the implementation of software (http://unafold.rna.albany.edu) revealing folded structures indicated that more complex folded structures of the CV amplicon could also account for lower amplification efficiency. (Fig. 5) Of note, in 4/38 (10.52%) of patients we observed significant differences in quantification between the two assays (114: $P = 0.00101$, 72: $P > 0.0001$, 23: $P > 0.0001$, 111: $P = 0.0003$) (Fig. 4A).

We next aimed to test the performance of the two assays when HIV-1 copy input was diluted to 10 copies. We found that in low copies, correlation was skewed towards the CV assay ($r = 0.8220$, $P < 0.0001$) and that in 9/25 (36%) of samples quantification was significantly different between the assays (Fig. 4D and F). However, there was no significant difference between the mean quantification of low copy patient samples ($P = 0.1456$) (Fig. 4E).

3.4. HIV-1 quantification using patient tailored primers

We showed that both assays performed comparably; however, there was discrepancy in quantification observed with some patient samples. We aimed to elucidate the cause of this discrepancy by sequencing the LTR of patient samples. Two forward and reverse primers were selected for nested LTR amplification based on identity with sequences of the Los Alamos database (Table 1). The LTR of patient samples was subsequently sequenced with the Sanger platform. Patient sequences were analysed using BioEdit and sequences were manually aligned to primer and probes used in both assays (data not shown). Based on this alignment, we selected primers tailored to patient samples (Table 1). Patient samples were quantified simultaneously with the universal and the patient tailored primers. For each patient sample tested, the quantification with patient tailored primers was significantly higher than when the universal primer was used (RvS 132 $p = 0.0056$, RvS 108 $p = 0.0083$, RvS 124 $p = 0.0010$, CV 132 $p = 0.0004$, CV 124 $p = 0.0008$, CV 132 $p = 0.0077$) (Fig. 6A and B). Together, this is a 131.9% and 141.6% average increase for RvS and CV assays, respectively, when patient tailored primers were utilised, demonstrating that sequence diversity can occasionally impair the accuracy of the assay.

We subsequently interrogated the sequence information available 'to date' at the Los Alamos HIV-1 database. Our in silico analysis revealed that the oligonucleotide with the higher propensity for mismatches was the RvS forward pre-amplification primer, at the 5′ end

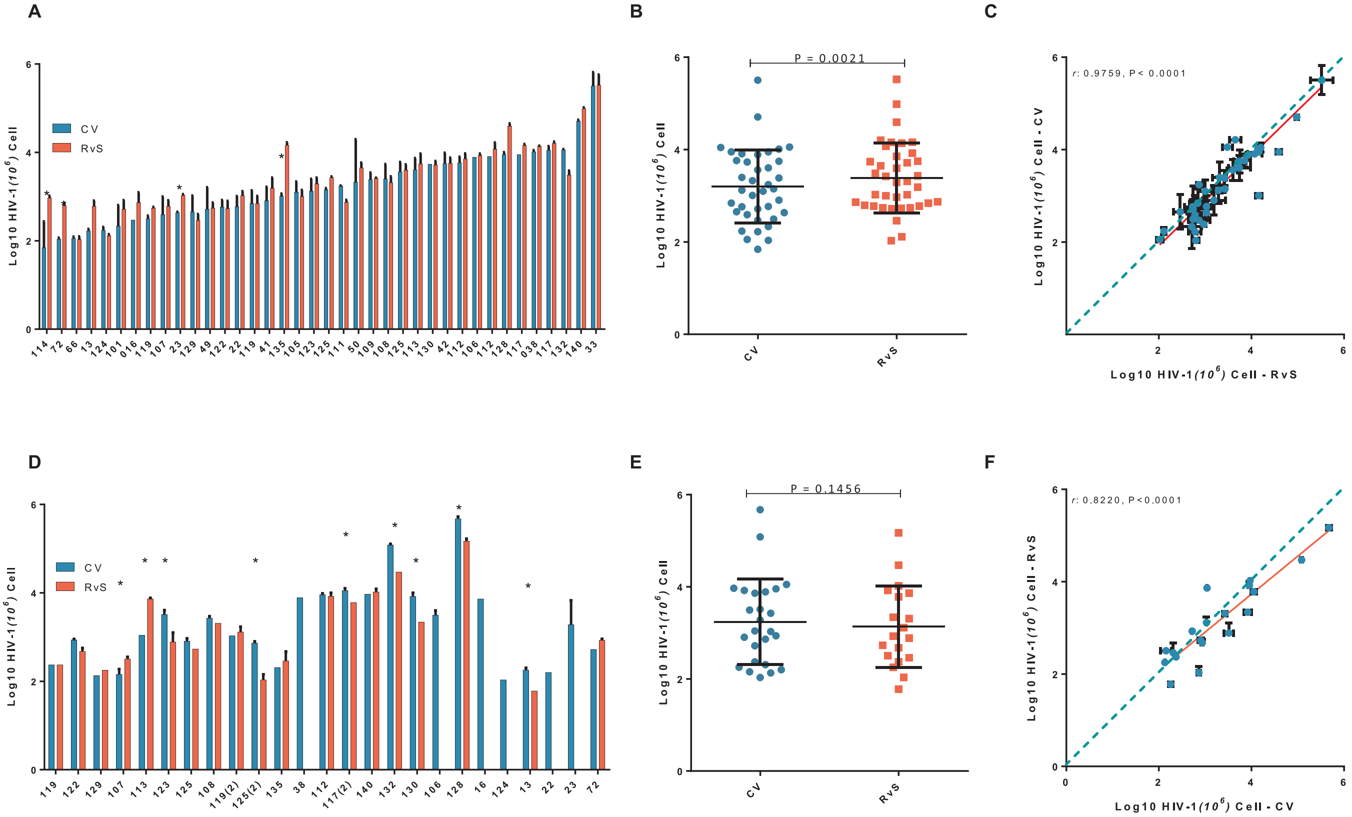

Fig. 4. Quantification of patient samples: A) Pellets of PBMCs or CD4+ cells extracted using proteinase K digestion or Qiagen DNA extraction. Total HIV-1 was quantified using RvS and CV assays and cells were quantified using the CD3 assay. Statistical significance determined using the multiple t-test, Holm-Sidak method, with alpha = 5.000%. B, E) Dot plot showing differences in mean quantification for undiluted and low copy quantification. Significance determined by paired t-test. D) Samples were diluted to 10 copies per reaction and quantified using both assays. Statistical significance determined using the multiple T test, Holm-Sidak method, with alpha = 5.000%. C, F) Correlation of all samples and correlation of diluted, low copy samples, respectively. Solid red line represents linear regression and green dashed line represents perfect correlation.

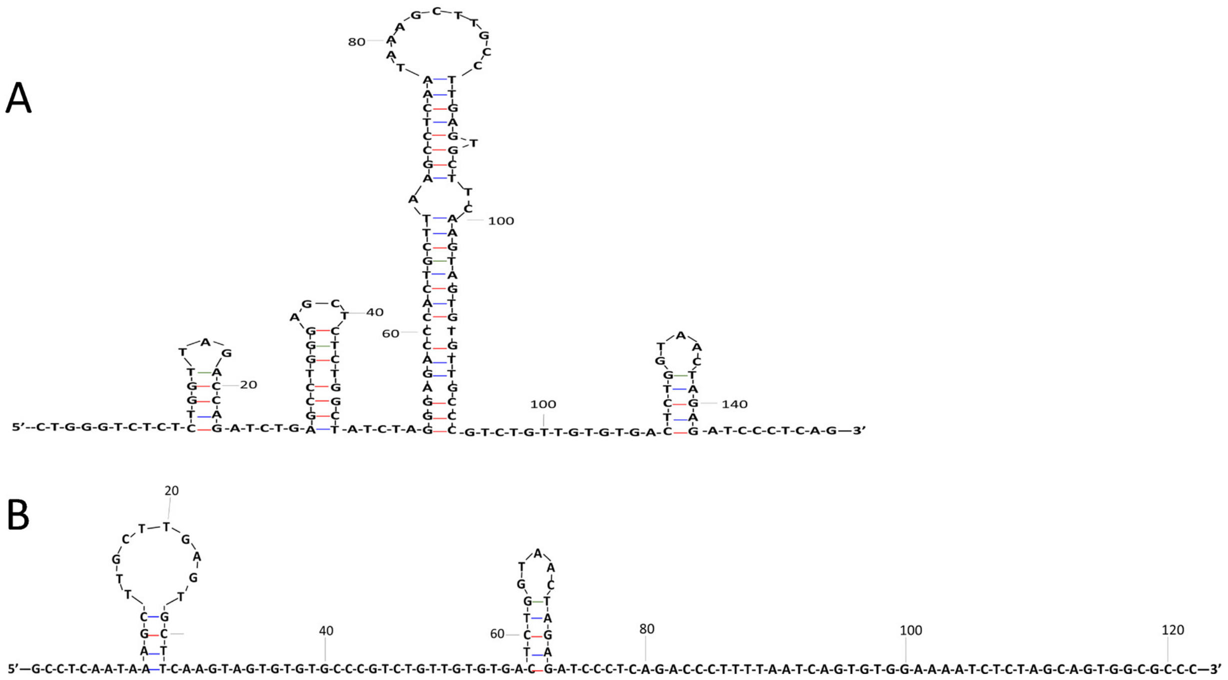

Fig. 5. The probable secondary structure of single stranded HIV-1 DNA produced using the The mfold Web Server (http://unafold.rna.albany.edu): A) Depicts the 152 nt CV amplicon (HxB2: 522→643) and B) Depicts the 122 nt RvS amplicon (HxB2: 452→603).

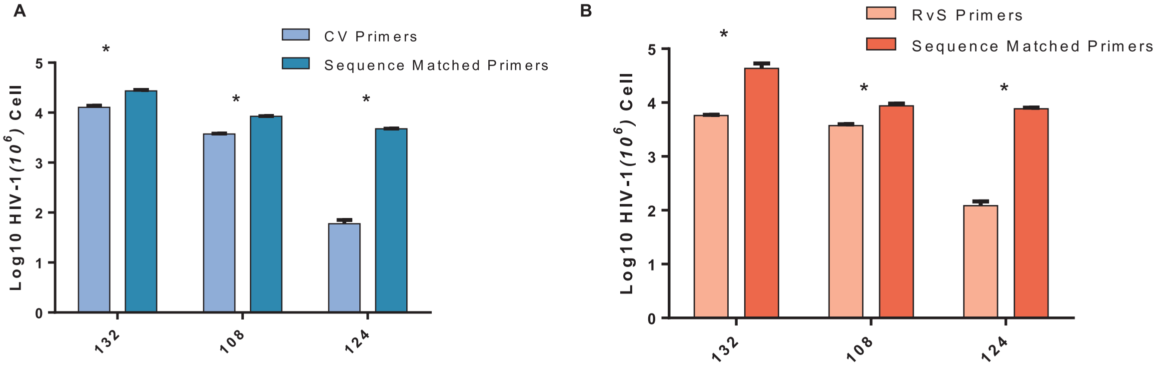

Fig. 6. Quantification with sequence matched primers: Primers designed to match sequences were compared with assay primers: A) Comparison of CV primers to sequence matched primers. B) Comparison of RvS primers to sequence matched primers. Statistical significance determined using the multiple *t*-test, Holm-Sidak method, with alpha = 5.000%.

(Fig. 7E), when compared to the other assay primers (Fig. 7A–D). We therefore redesigned this primer in two different versions (RvS-A and RvS-B, Table 1) (Fig. 7F), to compensate the sequence diversity and circumvent 5′ end mismatches that would be the most deleterious. These two primer versions were used at equal ratio for the pre-amplification step. Our results indicate that the new primers, RvS-A and RvS-B used in equal ratio, yield a significantly higher quantification than the existing primer, and this represents an improvement on the assay (P = 0.0057) (Fig. 8). Though this difference is small, our analysis suggests this primer combination will reduce the risk of mismatching in the 5′ end of the primer and increase the overall coverage and accuracy of the assay. As it stands the in silico analysis showed that the overall primer diversity ranged between 0.04% and 0.07% as estimated using the neighbour-joining method and the Kimura-2-parameter model (data not shown) suggesting that the RvS-A and B primer combination will rarely underestimate the total DNA load.

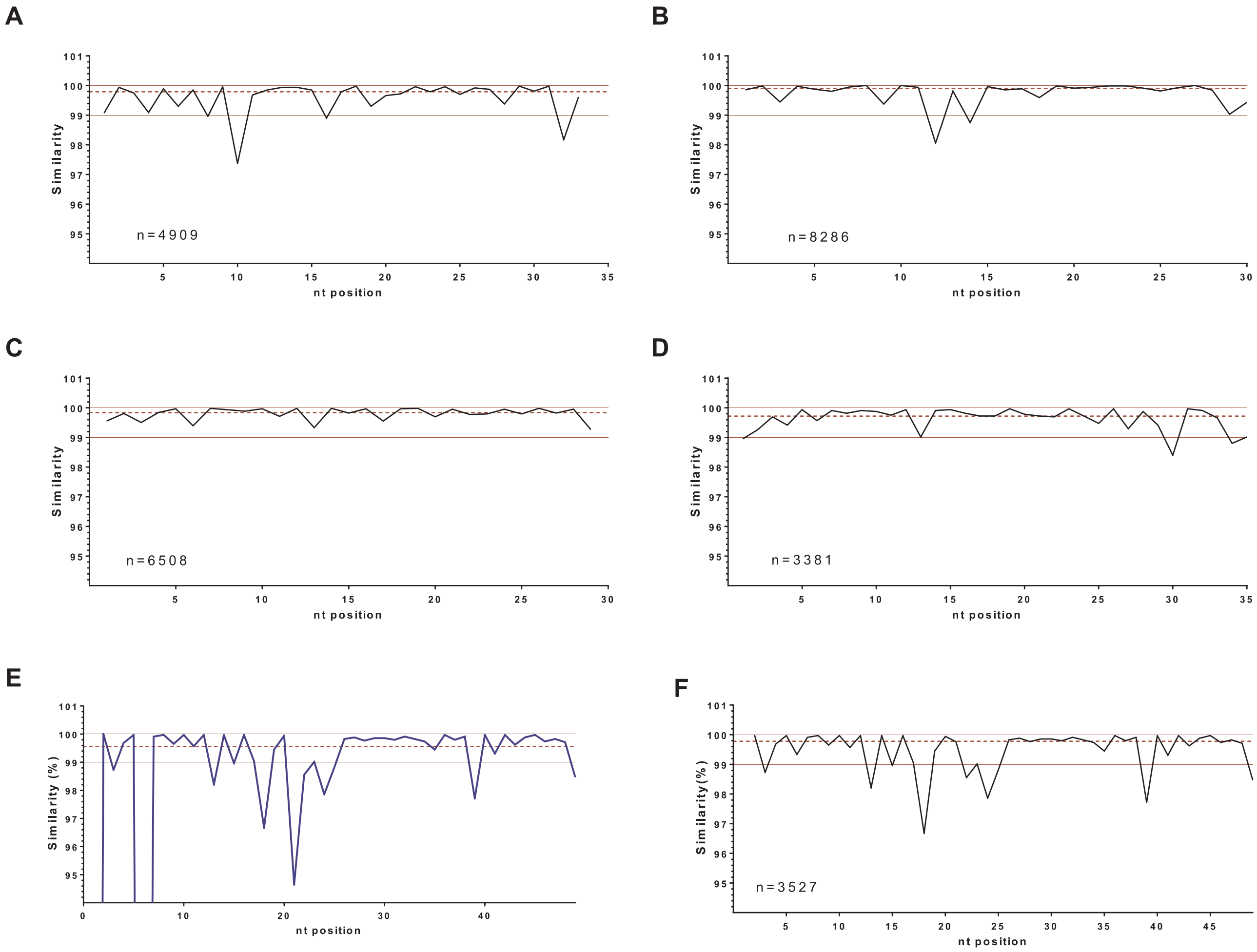

Fig. 7. Analysis of primer and probe sequence identity to published HIV-1 sequences: A) RvS probe sequence B) RvS pre-amplification reverse primer C) RvS qPCR reverse primer D) Region targeted by both VC Probe and RvS qPCR forward primer E) RvS pre-amplification forward primer F) Modified assay primer encompassing both primer A and B, where one has a nucleotide removed (Table 1) n = the number of sequences analysed per oligonucleotide.

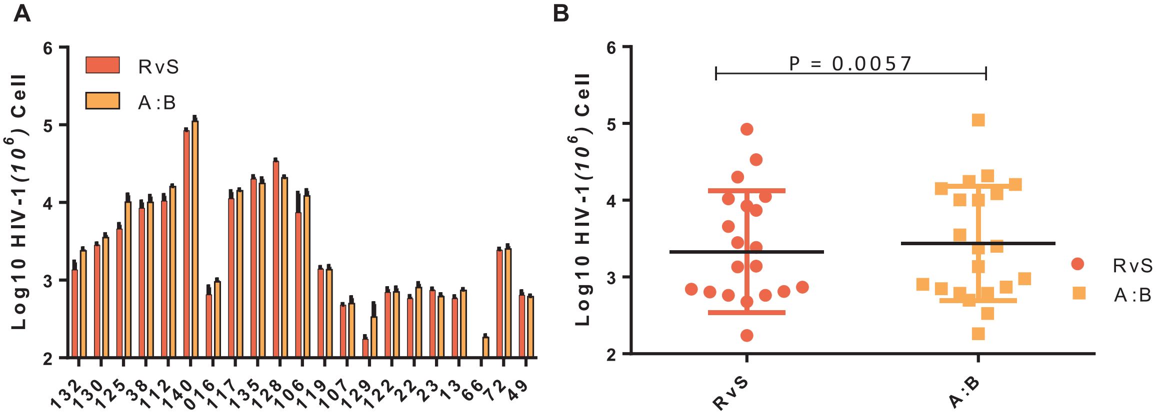

Fig. 8. Comparison of patient sample quantification when using the existing RvS forward primer (red) or a combination of newly designed forward primers, RvS-A and RvS-B (orange) (Table 1). RvS-A and RvS-B primers were used in equal ratio. Significance determined by paired t-test.

4. Discussion

Clinical trials assessing the efficacy of therapeutic vaccination or HIV-1 eradication strategies must utilise robust and reproducible HIV-1 DNA quantification assays. The lack of a standard quantification assay to measure total HIV-1 DNA has led to the development of a number of ‘in-house’ assays targeting different genomic regions for quantification, but this variation may render the results of different clinical trials incomparable. We selected two HIV-1 quantification assays, CV (Vandergeeten et al., 2014) and RvS (van der Sluis et al., 2013), for comprehensive evaluation to determine if results obtained were comparable and the assays could therefore be used interchangeably. These assays were selected for the ability to distinguish different HIV-1 DNA forms, including 2-LTR circular DNA, which can serve as a marker of recent infection and therefore be used to determine the success of treatment. The differential quantification of different DNA markers is facilitated by the use of a pre-amplification step and primers targeting the conserved regions of the LTR of the viral genome.

Recent data has suggested that 8E5, a commonly used latency model containing one copy of HIV-1 per cell, is unstable and rapidly loses HIV-1 copies during passaging (Busby et al., 2017; Wilburn et al., 2016). Further, a study has shown evidence of ongoing replication within ACH2 cells during passaging, resulting in an increase in HIV-1 copies per cell (Sunshine et al., 2016). This study has proposed the use of J-Lat cells as quantification standards as these contain a non-replication competent copy of HIV-1 that remains stable after a number of passages (Sunshine et al., 2016). Consistent with these findings, we have compared a number of well characterised calibration cell lines and discovered that 8E5 and ACH2 cells are unsuitable for use due to the change in HIV-1 DNA copies during passaging (Busby et al., 2017; Wilburn et al., 2016). Further, we have demonstrated that J-Lat cells contain ~1 copy per cell and would therefore be the most suitable for use in DNA quantification studies. The universal use of only one cell line as a calibration standard would reduce variability of different HIV-1 DNA assays and across different labs, rendering data obtained from studies and clinical trials more comparable. Further, we demonstrate that both LTR based assays amplify well-characterised HIV-1 calibration cell lines with equal efficiency, removing the potential of bias in quantification of patient samples arising from a bias in the amplification of the standard curve.

Our data indicate that both assays perform comparably when quantifying total HIV-1 DNA in patient samples as well as cell lines and that these quantifications correlate strongly. Despite this, we have shown that the RvS assay quantifies the patient set as a whole, 0.2 Log10 HIV-1 copies higher than the CV assay, suggesting that the quantification of patient samples is more efficient when using this assay. When these samples were diluted to ~10 copies per input the strength of the correlation of the assays was lost. This is due to inherently higher variation in the quantification of low copy samples, owing to the stochastic distribution of template within the sample. However, the assay was improved when primers were redesigned using sequences derived from a recent HIV-1 database.

The RvS and CV assays have the ability to only quantify HIV-1 DNA that has undergone full reverse transcription as both implement a pre-amplification step that utilises primers strategically placed to bind DNA only present following first and second strand transfer (Fig. 1). However, the RvS assay performed slightly better, possibly due to the smaller amplicon size. Based on that observation we improved the performance and accuracy of the RvS assay by undergoing an in-silico analysis of the primer sequences using all available HIV-1 sequences from the Los Alamos database. The high degree of HIV-1 sequence heterogeneity means that sequence variation will be encountered even within the most conserved regions of the genome. Our analysis showed that the forward pre-amplification primer was most divergent from published sequences and we therefore redesigned this primer and suggest that two primers (Table 1) should be used to improve the accuracy and sensitivity of this assay.

HIV-1 DNA quantification is an essential tool for monitoring HIV-1 vaccine and therapy trials due to its low cost, fast turnaround time and high throughput capacity. Notwithstanding its advantages, DNA based assays cannot distinguish between replication competent and replication defective pro-virus, and will therefore overestimate the size of the replication competent LR (Rouzioux and Avettand-Fenoël, 2018; Ruggiero et al., 2017). Despite this, recent studies have suggested defective pro-virus contributes to HIV-1 pathogenesis, and so measuring the size of all pro-virus present in a sample is useful marker of vaccine or treatment success and projection for disease progression (Rouzioux and Avettand-Fenoël, 2018; Ruggiero et al., 2017). In any case these described assays are a cheaper, faster and more practical alternative to the cell based viral outgrowth assay (VOA) which is able to specifically quantify only replication competent pro-virus by measuring virus production in PBMCs following activation (Rouzioux and Avettand-Fenoël, 2018). Here we demonstrate that whilst two HIV-1 quantification assays perform comparably we have improved the RvS assay through increasing the coverage of the diverse HIV-1 populations that can be detected with the assay.

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