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

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4 Running title: Robust method for HIV-1 DNA Quantification

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25 **Summary**

26 Currently there is no standard HIV-1 DNA quantification assay for use in clinical trials and a
27 comparison of HIV-1 assays has not been performed. Here, we evaluate two HIV-1 DNA
28 quantification assays for their ability to accurately quantify HIV-1 DNA load in patient samples.
29 Additionally, we have compared a number of commonly used HIV-1 latency cell for their use
30 as HIV-1 quantification standards. We have shown that the two assays perform comparably
31 and correlate strongly, however, one assay performs significantly better and this assay was
32 chosen for improvement. We have also shown that J-Lat are the most suitable HIV-1
33 quantification standard owing to the stability of the integrated viral genome. We have
34 redesigned primers based on analyses of the HIV-1 database to update and improve the HIV-
35 1 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 LTR (long terminal repeat) 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 and 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=0.0116, n=17).

Key Words: HIV-1 DNA quantification, latent reservoir, HIV-1 cure, HIV-1 therapeutic vaccines

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

73 **1. Introduction**

74 The development of active antiretroviral therapy (ART) has been a major breakthrough
75 in the treatment of human immunodeficiency virus type 1 (HIV-1) infection, effectively
76 preventing the progression to acquired immunodeficiency syndrome (AIDS) (Brechtl et al.,
77 2001). Despite this, ART cannot completely eradicate the virus due to the presence of a
78 replication competent latent reservoir (LR) in different cell populations including long-lived
79 resting CD4+ T cells that harbour pro-viral DNA integrated into the genome (Chun et al.,
80 1997a; Finzi et al., 1997). Such infected cells can produce replication competent HIV-1,
81 supporting rapid viral rebound following ART interruption (Davey et al., 1999; Joos et al.,
82 2008; Rothenberger et al., 2015). Research is therefore focused on the development of novel
83 approaches to reduce or eliminate the LR, with the aim of developing a functional cure for
84 HIV-1 infection.

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

97 Alternatively, strategies which aim to completely eradicate the HIV-1 LR are popular in current
98 research and clinical trials (Kim, Anderson, and Lewin, 2018). These “shock and kill”
99 approaches utilise latency reversing agents (LRA) to induce activation of latently infected cells
100 in the presence of ART, rendering those cells susceptible to cytolysis or immune clearance
101 whilst limiting the chance of subsequent rounds of infection (Archin et al., 2017; Archin et al.,
102 2012; Elliott et al., 2015; Margolis et al., 2016). Adding to this, recent approaches have
103 explored the potential of a “lock in and apoptosis” strategy that when combined with the
104 LRAs, utilises a novel compound to antagonise the viral gag protein and prevent virus budding
105 whilst still inducing virus apoptosis (Tateishi et al., 2017).
106 Research focused on the reduction or elimination of the LR must utilise robust assays
107 that can reliably and reproducibly measure the effect that the treatment or vaccine strategy
108 has on the size of the LR. The quantification of HIV-1 DNA from peripheral blood mononuclear
109 cells (PBMC) of patients via polymerase chain reaction (PCR) provides a useful tool to monitor
110 the size of the viral reservoir, and a number of assays have been developed targeting different
111 regions of the HIV-1 genome including gag, *pol* and the long terminal repeat (LTR) (Beloukas
112 et al., 2009; Kabamba-Mukadi et al., 2005; Rouzioux and Avettand-Fenoël, 2018; van der Sluis
113 et al., 2013; Vandergeeten et al., 2014; Yun, Fredriksson, and Sonnerborg, 2002). The strength
114 of these assays is the rapid turn-around from sample collection to DNA quantification and the
115 possibility to identify different HIV-1 DNA forms, such as integrated DNA, unintegrated linear
116 DNA forms and 2-LTR circular DNA (De Spiegelaere et al., 2014; van der Sluis et al., 2013;
117 Vandergeeten et al., 2014). These different HIV-1 DNA forms have been used as markers of
118 HIV-1 persistence in a number of different studies, reviewed here (Ruggiero et al., 2017). 2-
119 LTR circular DNA is a product of abortive integration, and while some studies have suggested
120 they are stable in CD4+ cells (Pace, Graf, and O'Doherty, 2013), they are considered markers

121 of recent infection and ongoing replication notwithstanding therapy (Chun et al., 1997b;
122 Koelsch et al., 2008; Zhu et al., 2011). Only assays targeting the viral LTR allow for the
123 discrimination of different HIV-1 forms in addition to the fact that the LTR contains some of
124 the most conserved regions of the viral genome (van der Sluis et al., 2013). We have
125 comprehensively analysed two HIV-1 DNA quantification assays, herein referred to as
126 Vandergeetan, (CV) (Vandergeeten et al., 2014) and van der Sluis (RvS) (van der Sluis et al.,
127 2013), both of which target highly conserved regions in the long terminal repeat (LTR) region
128 of the virus genome (van der Sluis et al., 2013; Vandergeeten et al., 2014) (Fig. 1). Both assays
129 utilise a PCR pre-amplification step with primers designed to only amplify DNA which has been
130 fully reverse transcribed, excluding the short abortive transcripts. Additionally, both assays
131 are able to distinguish between total HIV-1 DNA and 2-LTR circular DNA with the use of
132 alternative primer sets in the pre-amplification step. The CV assay is also able to distinguish
133 integrated HIV-1 DNA via the use of primers targeting human *Alu* sequences, randomly
134 dispersed in the human genome (Ruggiero et al., 2017; Vandergeeten et al., 2014). A
135 prominent HIV-1 LTR based DNA assay, herein referred to as Rouzioux (CRx), was excluded
136 from this comparison because this assay does not distinguish between different DNA types
137 (Rouzioux, Melard, and Avettand-Fenoel, 2014). Furthermore, we have evaluated several
138 calibration cell-lines, aiming to establish a stable and reproducible source of HIV-1 DNA for
139 use as a standard curve. Additionally, we have analysed the primer sequences and used this
140 information to establish an assay with broader specificity and increased sensitivity.
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144 **2. Materials and Methods**

145 **2.1. Cell Lines and Calibration Standards.** HIV-1 quantification standards were produced from
146 cell lines including 8E5 (CFAR 95), ACH-2 (CFAR 349) and J-Lat 10.6 (CFAR 9849), obtained
147 from the NIH AIDS reagent program. Additionally, we utilised SupT1-14 a previously
148 characterised cell lined containing 14 HIV-1 copies per cell in comparing the assays (van der
149 Sluis et al., 2013). Standards for the quantification of cell input were produced from dilutions
150 of DNA derived from HEK293T cells (ATCC CRL-3216). ACH-2, 8E5 and J-Lat 10.6 were
151 maintained in RPMI-1640 medium (Fisher, 11875093) supplemented with 10% heat
152 inactivated FBS (Sigma, non-US origin, F7524) and 1% pen-strep (Fisher, 15140122) at 37 °C
153 with 5% CO_2. HEK293T cells were maintained under the same conditions with advanced
154 DMEM (Fisher, 12491015) used for culturing. Cells were passaged to a maximum of 10 cycles
155 prior to DNA extraction using QIAamp DNA Blood Mini Kit, according to the manufacturer's
156 instructions (Qiagen, 51104). DNA concentration and purity was assessed by Nanodrop
157 analysis (Thermo Scientific, ND-200). The total number of cells and HIV-1 copy numbers were
158 quantified using the CD3 and LTR quantification assays, respectively, and as previously
159 described (van der Sluis et al., 2013; Vandergeeten et al., 2014). Standards were produced via
160 a dilution series over a range of 5 logs. HIV-1 DNA standards were spiked with uninfected
161 human genomic DNA to equalise DNA input in lower copy numbers.
162
163 **2.2. Study Population Clinical Sample Preparation.** The present study was approved by the
164 Institutional Review Board of the Centre Hospitalier Universitaire Vaudois and all subjects
165 provided written informed consent. CD4+ cells were isolated from PBMCs by negative
166 selection using paramagnetic beads (StemCell Technologies) according to supplier's protocol.
167 Purified CD4+ cells were digested via incubation with 0.1 mg/ml recombinant proteinase K
168 (Roche, RPROTK-RO) in 10 nM Tris (pH 8.3) for 2 h at 56 °C. Lysate was centrifuged and

169 supernatant recovered and used as input in the HIV-1 quantification assays. Proteinase K
170 lysates were stored at -80 °C until use.
171
172 **2.3. HIV-1 DNA Quantification Assays.** Total HIV-1 DNA was quantified using both CV
173 (Vandergeeten et al., 2014) and RvS (van der Sluis et al., 2013) LTR based HIV-1 DNA as well
174 as CD3 cell quantification assays. Primers and probes used in HIV-1 and CD3 DNA
175 quantification are as described previously (van der Sluis et al., 2013; Vandergeeten et al.,
176 2014). Additionally, a commercial cell quantification kit targeting the β-actin gene was used
177 (ABI Applied Biosystems TaqMan β-actin Detection Reagent, 401846). Pre-amplifications
178 were performed in 25 µl reactions using Taq polymerase (Invitrogen, 10342020), as previously
179 described (van der Sluis et al., 2013; Vandergeeten et al., 2014). Quantifications were
180 performed in 20 µl reactions using Supermix-UDG (Invitrogen, 11730025) with the Qiagen
181 Rotor Gene RotorQ, as described previously (van der Sluis et al., 2013; Vandergeeten et al.,
182 2014). β-actin quantifications were performed according to the manufacturer's instructions.
183 Reagent mixes for the quantification and pre-amplification PCR steps were adapted to the
184 volumes used in this study, though the final concentrations remained the same as previously
185 described (van der Sluis et al., 2013; Vandergeeten et al., 2014).
186
187 **2.4. Sanger DNA Sequencing.** To sequence the primer and probe binding regions of both
188 assays primers were designed in house to amplify the LTR region of patient samples (Table 1).
189 Nested PCR was performed under the following conditions: 2 min (95 °C) followed by 35 cycles
190 of 30 s (95 °C), 30 s (55 °C) and 1 min (72 ◦C) with a final elongation of 10 min (75 °C). The
191 product of PCR 1 was diluted 1/10 in molecular grade water and this dilution was

192 subsequently used as input for PCR 2. Amplification was analysed using gel electrophoresis

193 and further purified using a Qiagen PCR Purification Kit (28104) prior to sequencing (GATC

194 Biotech and Source Bioscience). Patient sequences were then aligned to primer and probe

195 sequences using BioEdit software to identify mismatches. Following this, new primers were

196 selected to exactly match the patient sample LTR region and used to quantify the total HIV-1

197 DNA using both assays, as described above (Table 1).

198

199 **3. Results**

200 **3.1. Validation of assay quantification standards.** Our aim was to examine the performance

201 of the two assays CV and RvS and using the vast amount of sequence information available to

202 date develop a new assay that will perform most optimally with the highest specificity and

203 sensitivity. The incentive for this consideration was that both HIV-1 DNA quantification assays

204 target the LTR of the HIV-1 genome, well established as the most conserved region of the

205 genome, and furthermore both utilise a pre-amplification step allowing for the separate

206 quantification of different viral life-cycle stages. In order to do so we initially aimed to define

207 the cell quantification standard using a human genomic DNA input based on 293T cells. We

208 quantified the cell number using two methods; a commercial assay with primers targeting the

209 human β-actin gene and a previously described assay targeting the human CD3 gene

210 (Vandergeeten et al., 2014). We tested a 5 log standard range (10^5 to 10^1 cell equivalents)

211 using both assays and found that they were within the optimum range of amplification

212 efficiency (90-110%) and that there was no significant difference between either over 3 runs

213 (P=0.8538) (Fig. 2A and 2B). Based on this result we selected the CD3 quantification assay

214 because it includes a pre-amplification step consistent with the HIV-1 DNA assays.

215 Further, we ran the two HIV-1 quantifications assays, RvS (van der Sluis et al., 2013) and
216 CV (Vandergeeten et al., 2014), using 5-log serial dilutions (10^5 to 10^1 HIV-1 copies per input)
217 of the J-Lat 10.6, 8E5, SupT-14 and ACH2 cell lines. We found no significant difference
218 between qPCR efficiency of both assays over 6 runs (P=0.0552). We next compared the HIV-
219 1 DNA content in these cell lines, aiming to determine the most appropriate cell line for use
220 as a quantification standard.
221

222 **3.2 Evaluation of calibration cell lines.** We evaluated HIV-1 integration model cell lines
223 including ACH2, 8E5 and J-Lat as well as in 'in house' cell line, SupT-14, for their use as
224 calibration standards. Cell lines were grown to 10 passages and the total HIV-1 per 10^6 cells
225 was quantified following DNA extraction. Consistent with recent publications, we showed that
226 HIV-1 copies per cell decreased in 8E5 cells from 1 to 0.2 copies (Fig. 3). Additionally, HIV-1
227 copies in ACH2 cells were found to increase from 1 to 4 copies per cell. On the contrary, HIV-
228 1 DNA content was stable in both J-Lat 10.6 and SupT1-14, which contain 1 copy per cell
229 consistent with recent studies (Sunshine et al., 2016) and 14 copies per cell as demonstrated
230 previously (van der Sluis et al., 2013), respectively (Fig. 3). Based on these findings we used
231 the J-Lat 10.6 to quantify patient samples in this study.
232

233 **3.3 HIV-1 quantification in patient samples.** We then compared the two HIV-1 DNA
234 quantification assays using patient samples. Overall, a strong correlation was found between
235 the results produced with the RvS and CV assays (*r*=0.9759, P<0.0001) (Fig. 4C). Nonetheless,
236 the mean quantification of patient samples was significantly higher when using the RvS (3.385
237 Log10 HIV-1 copies/106 cells) HIV-1 assay compared to the CV assay (3.203 Log10 HIV-1
238 copies/106) (P=0.0021) suggesting a slight advantage of RvS over CV when testing patient

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

246 We next aimed to test the performance of the two assays when HIV-1 copy input was
247 diluted down to 10 copies. We found that in low copies, correlation was skewed towards the
248 CV assay (*r*=0.8220, P<0.0001) and that in 9/25 (36%) of samples quantification was
249 significantly different between the assays (Fig. 4D and 4F). However, there was no significant
250 difference between the mean quantification of low copy patient samples (P=0.1456) (Fig. 4E).
251

252 **3.4 HIV-1 quantification using patient tailored primers.** We showed that both assays perform
253 comparably; however, there is some discrepancy in quantification observed with some
254 patient samples. We aimed to elucidate the cause of this discrepancy by sequencing the LTR
255 of patient samples. Two forward and reverse primers were selected for nested LTR
256 amplification based on identity with sequences of the Los Alamos database (Table 1). The LTR
257 of patient samples was subsequently sequenced with the Sanger platform. Patient sequences
258 were analysed using BioEdit and sequences were manually aligned to primer and probes used
259 in both assays (data not shown). Based on this alignment, we selected primers tailored to
260 patient samples (Table 1). Patient samples were quantified simultaneously with the universal
261 and the patient tailored primers. The HIV-1 DNA copy numbers were significantly increased
262 (131.9% and 141.6% average increase for RvS and CV assays, respectively) when matching

263 primers were used (Fig. 6A and 6B) with the corresponding patient samples demonstrating
264 that sequence diversity can occasionally impair the accuracy of the assay.

265 We subsequently interrogated the sequence information available 'to date' at the Los
266 Alamos HIV-1 database. Our in silico analysis revealed that the oligonucleotide with the higher
267 propensity for mismatches was the RvS forward pre-amplification primer, at the 5' end (Fig.
268 7E), when compared to the other assay primers (Fig. 7A-D). We therefore redesigned this
269 primer in two different versions (RvS-PreF-A and RvS-PreF-B, Table 1) (Fig. 7F), to compensate
270 the sequence diversity and circumvent 5' end mismatches that would be the most
271 deleterious. These two primer versions were used at equal ratio for the pre-amplification
272 step. Our results indicate that the new primers yield a significantly higher quantification than
273 the existing primer, and this represents and improvement on the assay (P=0.0116) (Fig. 8).
274 Though this difference is small, our analysis suggests this primer combination will reduce the
275 risk of mismatching in the 5' end of the primer and increase the overall coverage and accuracy
276 of the assay. As it stands the *in silico* analysis showed that the overall primer diversity ranged
277 between 0.04% and 0.07% as estimated using the neighbour-joining method and the Kimura-
278 2-parameter model (data not shown) suggesting that the RvS primer combination will rarely
279 underestimate the total DNA load.

280

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283 **4. Discussion**

284 Clinical trials assessing the efficacy of therapeutic vaccination or HIV-1 eradication
285 strategies must utilise robust and reproducible HIV-1 DNA quantification assays. The lack of a
286 standard quantification assay to measure total HIV-1 DNA has led to the development of a

287 number of ‘in-house’ assays targeting different genomic regions for quantification, but this
288 variation may render the results of different clinical trials incomparable. We selected two HIV-
289 1 quantification assays, CV (Vandergeeten et al., 2014) and RvS (van der Sluis et al., 2013), for
290 comprehensive evaluation to determine if results obtained were comparable and the assays
291 could therefore be used interchangeably. These assays were selected for the ability to
292 distinguish different HIV-1 DNA forms, including 2-LTR circular DNA, which can serve as a
293 marker of recent infection and therefore be used to determine the success of treatment. The
294 differential quantification of different DNA markers is facilitated by the use of a pre-
295 amplification step and primers targeting the conserved regions of the LTR of the viral genome.
296 Recent data has suggested that 8E5, a commonly used latency model containing one
297 copy of HIV-1 per cell, is unstable and rapidly loses HIV-1 copies during passaging (Busby et
298 al., 2017; Wilburn et al., 2016). Further, a study has shown eveidence of ongoing replication
299 within ACH2 cells during passaging, resulting in an increase in HIV-1 copies per cell (Sunshine
300 et al., 2016). This study has proposed the use of J-Lat cells as quantification standards as these
301 contain a non-replication competent copy of HIV-1 that remains stable after a number of
302 passages (Sunshine et al., 2016). Consistent with these findings, we have compared a number
303 of well characterised calibration cell lines and discovered that 8E5 and ACH2 cells are
304 unsuitable for use due to the change in HIV-1 DNA copies during passaging (Busby et al., 2017;
305 Wilburn et al., 2016). Further, we have demonstrated that J-Lat cells contain ~1 copy per cell
306 and would therefore be the most suitable for used in DNA quantification studies. The
307 universal use of only one cell line as a calibration standard would reduce variability of
308 different HIV-1 DNA assays and across different labs, rendering data obtained from studies
309 and clinical trials more comparable. Further, we demonstrate that both LTR based assays
310 amplify well-characterised HIV-1 calibration cell lines with equal efficiency, removing the

311 potential of bias in quantification of patient samples arising from a bias in the amplification
312 of the standard curve.

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

322 The RvS assay utilises primers strategically placed to bind DNA only present following
323 first and second strand transfer of the reverse transcription process, ensuring that only fully
324 reverse transcribed DNA is amplified and therefore increasing the specificity and accuracy of
325 the assay (Figure 1) (van der Sluis et al., 2013). Based on this property we decided to improve
326 the assay using an analysis of sequences from the HIV-1 database. The high degree of HIV-1
327 sequence heterogeneity means that sequence variation will be encountered even within the
328 most conserved regions of the genome. Our analysis showed that the forward pre-
329 amplification primer was most divergent from published sequences and we therefore
330 redesigned this primer and suggest that two primers (Table 1) should be used to improve the
331 accuracy and sensitivity of this assay.

332 HIV-1 DNA quantification is an essential tool for monitoring HIV-1 vaccine and therapy
333 trials due to its low cost, fast turnaround time and high throughput capacity. Notwithstanding
334 its advantages, DNA based assays cannot distinguish between replication competent and

335 replication defective pro-virus, and will therefore overestimate the size of the replication
336 competent LR (Rouzioux and Avettand-Fenoël, 2018; Ruggiero et al., 2017). Despite this,
337 recent studies have suggested defective pro-virus contributes to HIV-1 pathogenesis, and so
338 measuring the size of all pro-virus present in a sample is useful marker of vaccine or treatment
339 success and projection for disease progression (Rouzioux and Avettand-Fenoël, 2018;
340 Ruggiero et al., 2017). In any case these described assays are a cheaper, faster and more
341 practical alternative to the cell based viral outgrowth assay (VOA) which is able to specifically
342 quantify only replication competent pro-virus by measuring virus production in PBMCs
343 following activation (Rouzioux and Avettand-Fenoël, 2018). Here we demonstrate that two
344 HIV-1 quantification assays perform comparably and can therefore be used interchangeably
345 in clinical settings. Furthermore, we have improved the RvS assay through increasing the
346 coverage of the diverse HIV-1 populations that can be detected with the assay.
347

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352

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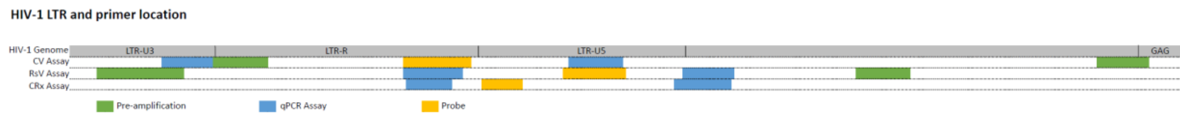
506 **FIGURE LEGENDS**

507 **Fig. 1.** HIV-1 LTR region: Locations of primers and probes for the CV, RvS and CRx assays. The

508 numbers indicate the position on the HXB2 genome. For CV assay forward quantification

509 primer anneals to Lambda T heel sequence on the forward pre-amplification primer.



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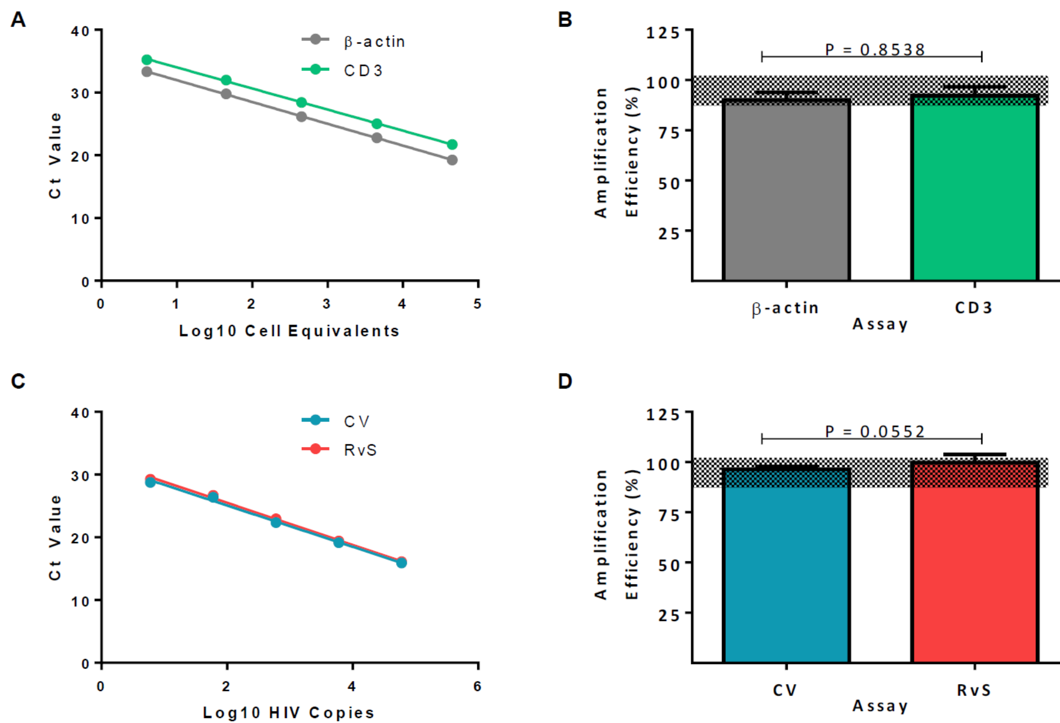
511 **Fig. 1.** Comparison of standards and assay performance: A) 5 log serial dilution of human

512 genomic DNA quantified using CD3 and β-actin qPCR. B) Average amplification efficiency of

513 CD3 and β-actin assays (n=3). C) 5 log serial dilution of J-Lat clone 10.6 cells using CV and RvS.

514 D) Average amplification efficiency of CV and RvS assays.



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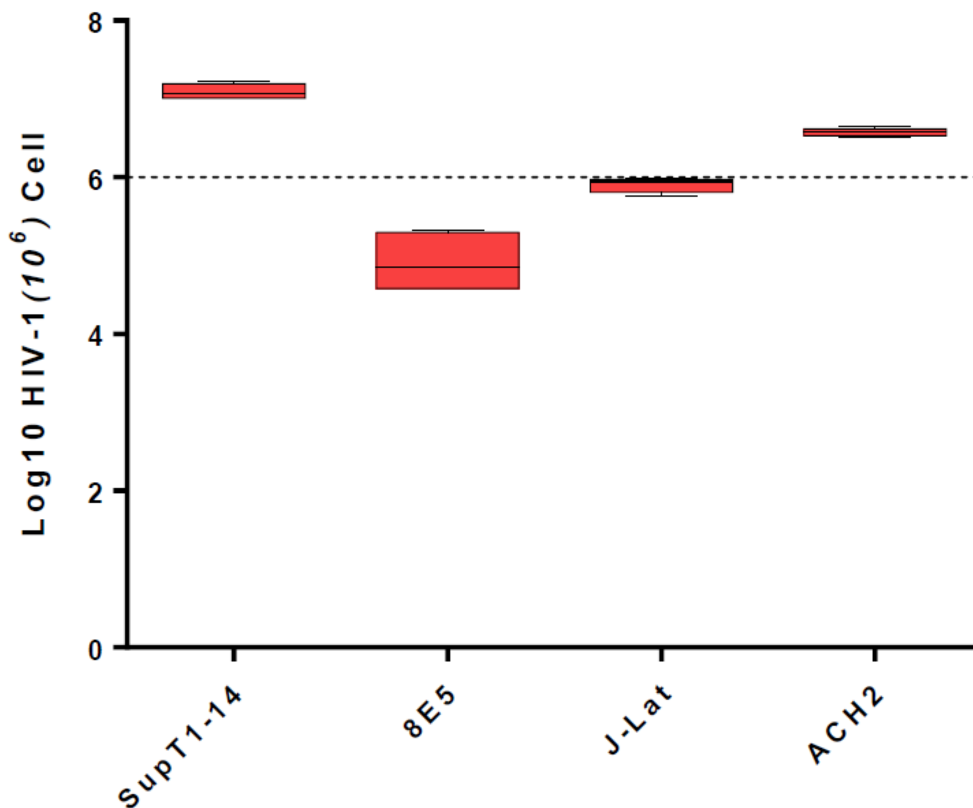
516 **Fig. 3.** Quantification using different cell lines: Quantification of three cell lines containing 1

517 HIV-1 copy per cell (8E5, J-Lat) and 14 HIV-1 copies per cell (SupT1-14). A five log dilution

518 series for each cell line was performed and used as input for the assay. Quantification of each

519 dilution was pooled and standardised to determine the average HIV-1 copies per cell.



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

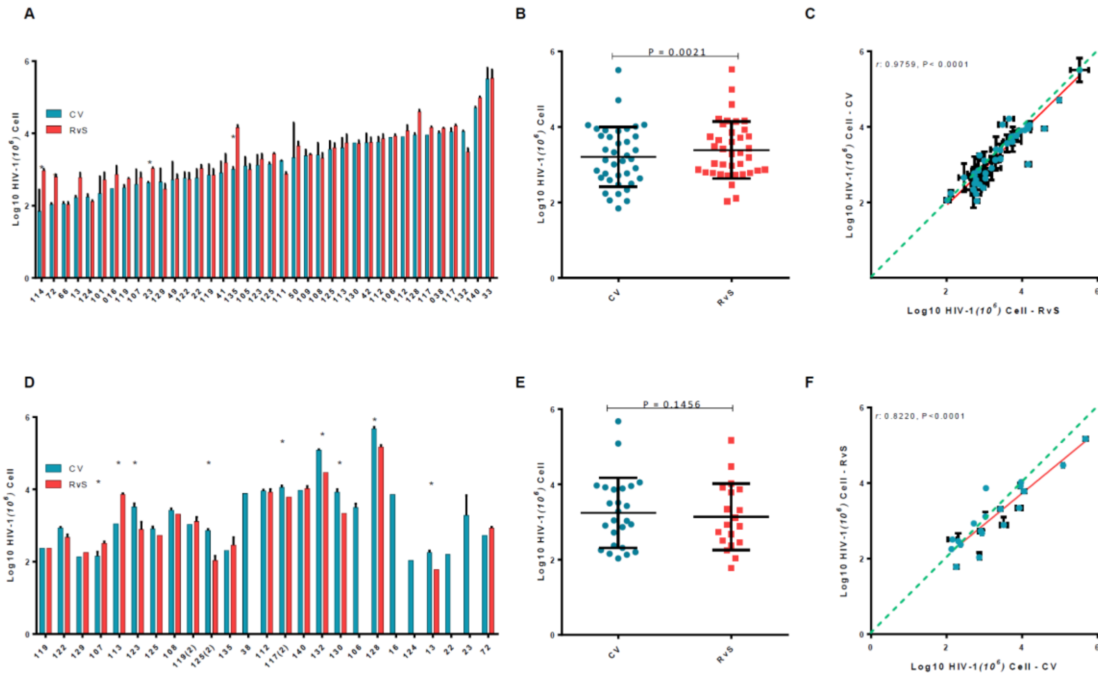


Fig. 5. The probable secondary structure of single stranded HIV-1 DNA produced using the The mfold Web Server (http://unafold.rna.albany.edu): A) Depicts the 152 nt CV amplicon (HxB2: 522→643) and B) Depicts the 122 nt RvS amplicon (HxB2: 452→603).

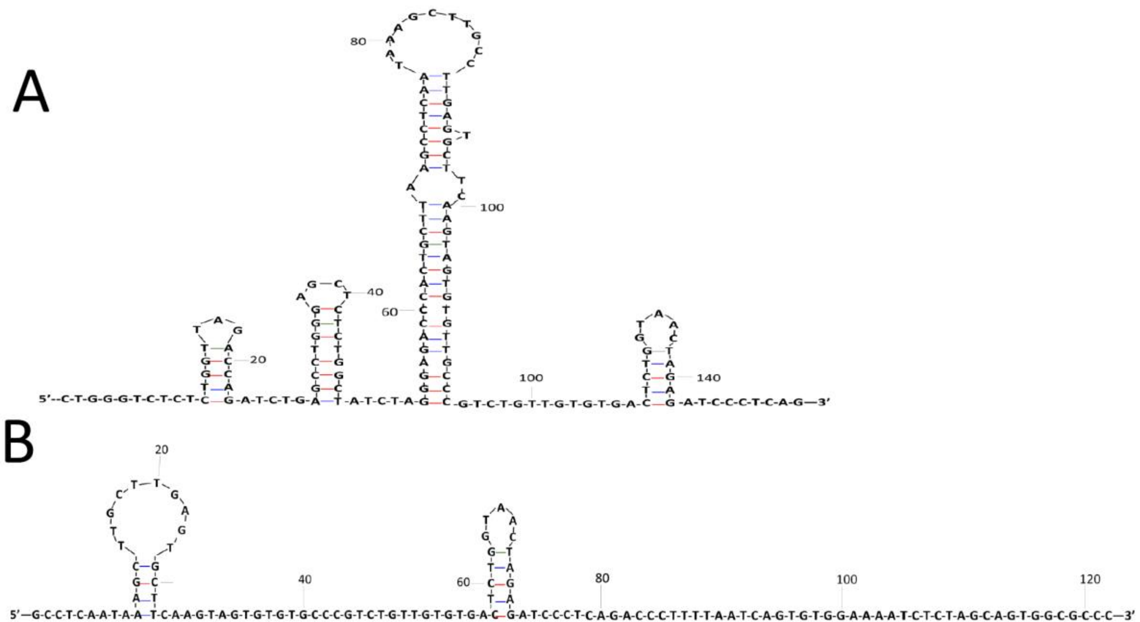
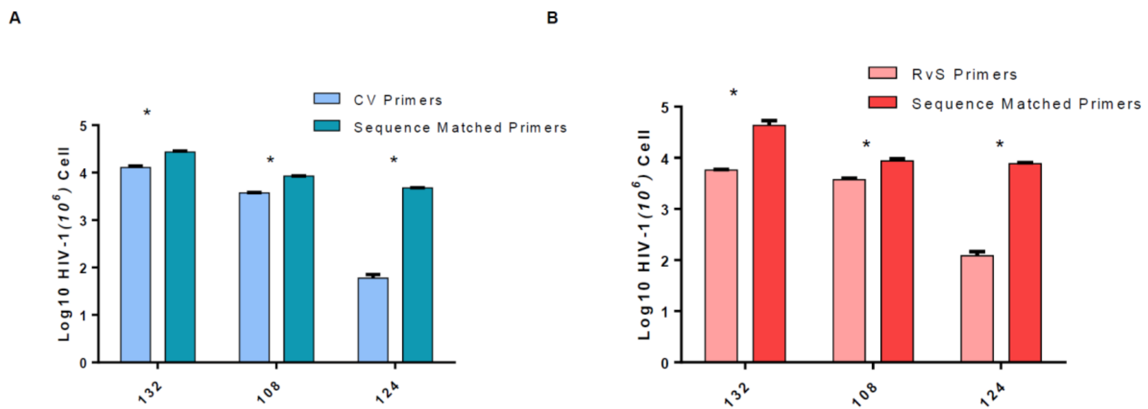


Fig. 6. Quantification with sequence matched primers: Primers designed to match sequences were compared with assay primers: A) Comparison of CV primers to sequence matched

538 primers. B) Comparison of RvS primers to sequence matched primers. Statistical significance

539 determined using the multiple t-test, Holm-Sidak method, with alpha = 5.000%.



540

541 **Fig. 7.** Analysis of primer and probe sequence identity to published HIV-1 sequences: A) RvS

542 probe sequence B) RvS pre-amplification reverse primer C) RvS qPCR reverse primer D) Region

543 targeted by both VC Probe and RvS qPCR forward primer E) RvS pre-amplification forward

544 primer F) Modified assay primer encompassing both primer A and B, where one has a

545 nucleotide removed (Table 1) n = the number of sequences analysed per oligonucleotide.

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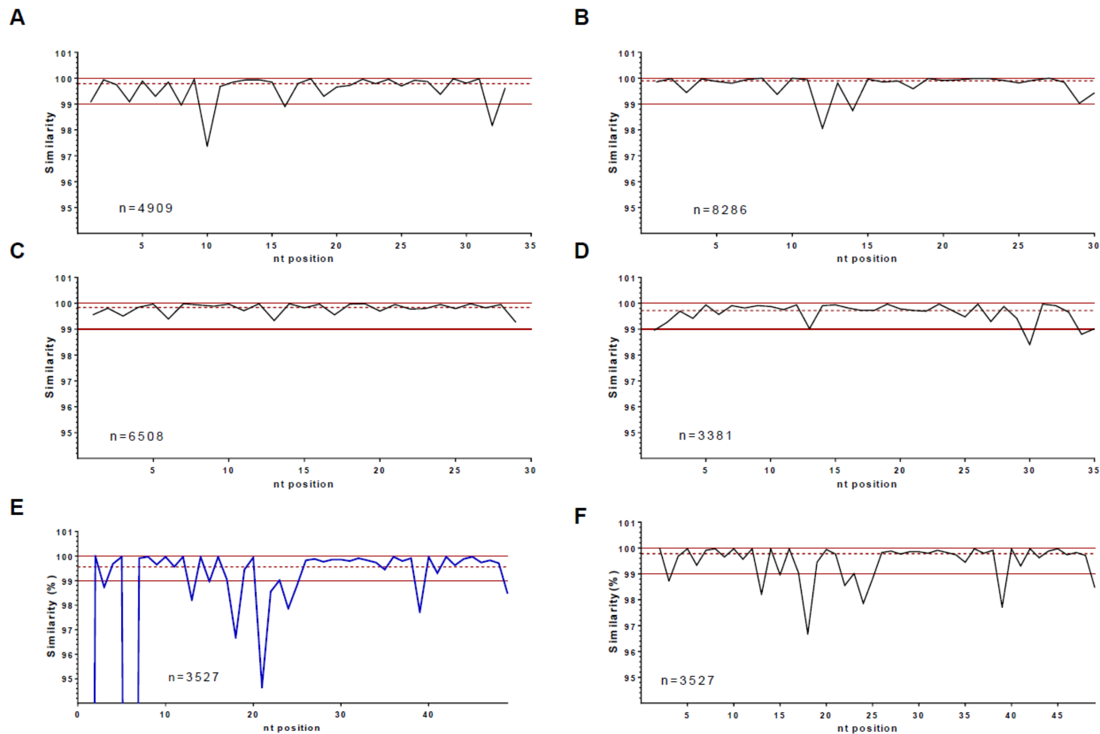

547 **Fig. 8.** Comparison of new compared to existing primers to quantify patient samples. Samples

548 were quantified with an equal mix of primer A and B (Table 1) to increase coverage of the

549 genome. Significance determined by paired t-test.

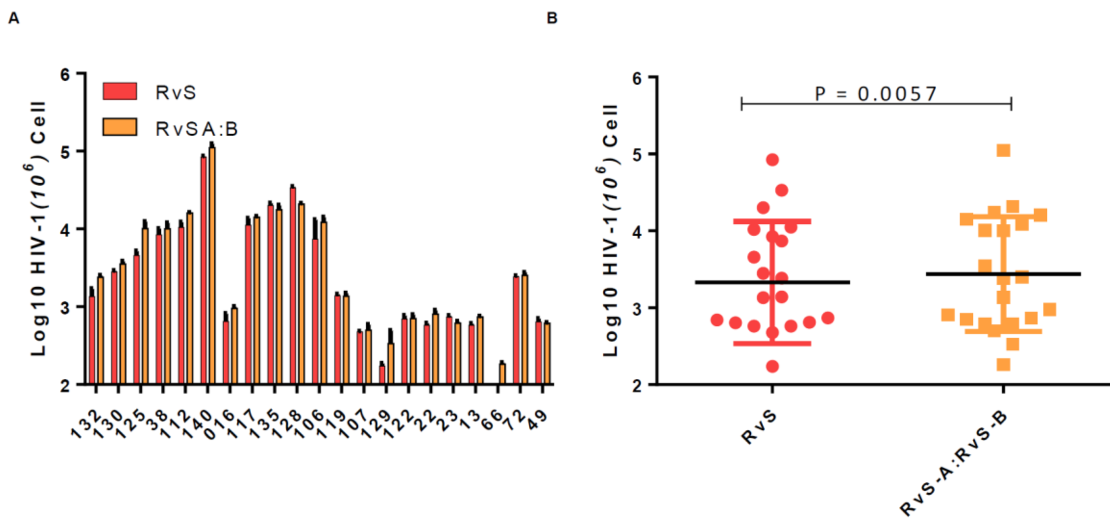

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TABLE 1 Primers used for the amplification of patient sample LTRs and primers matched to patient sequences. For tailored and redesigned primers nucleotide positions that vary from the universal assay primer are underlined.

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