HIV-1 latency in proliferating T cells
van der Sluis, R.M.

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Chapter Two

Quantitation of HIV-1 DNA
with a sensitive TaqMan assay that has broad subtype specificity.

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ABSTRACT

The increasing diversity of HIV-1 isolates makes virus quantitation challenging, especially when diverse isolates co-circulate in a geographical area. Measuring the HIV-1 DNA levels in cells has become a valuable practical tool for fundamental and clinical research. A quantitative HIV-1 DNA assay was developed based on TaqMan technology. Primers that target the highly conserved LTR region were designed to detect a broad array of HIV-1 variants, including viral isolates from many subtypes, with high sensitivity. Introduction of a pre-amplification step prior to the TaqMan reaction allowed the specific amplification of fully reverse transcribed viral DNA. Execution of the pre-amplification step with a second primer set enables for the exclusive quantitation of the 2-LTR circular HIV-1 DNA form.
INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) isolates can be classified in four different groups: Major (M), New (N), Outlier (O) and P, with group M viruses being mainly responsible for the pandemic\(^1-3\). The M group is divided into subtypes A through K and contains more than 51 circulating recombinant forms (CRFs) that are distributed unequally over the world\(^4\). The subtype B viral isolate is found mainly in Western Europe and North America. In Thailand, on the contrary, the recombinant AE (CRF_01) subtype is dominant. Many different HIV-1 subtypes and CRFs circulate in Africa but subtype C viruses dominate in the Sub-Saharan area and the recombinant AG (CRF_02) prevails in West and West-Central Africa\(^5-7\).

Over the years anti-viral reagents and viral detection assays have been developed primarily for European and American subtype B viruses. This bias originates from the fact that this was the first subtype isolated in the Western world and used for the development of viral reagents and assays. Currently, subtype B viruses account for 10% of all infections worldwide, whereas 50% of the infected people carry a subtype C virus\(^5,8\). Most HIV-1 quantitation assays used in a clinical setting and the majority of drugs used in combined antiretroviral therapy (cART) have been developed against subtype B viruses. With the introduction of therapy in Africa it is important to determine whether or not cART inhibits other subtypes with similar efficiency as subtype B. For instance, two previous reports have demonstrated that subtype C harbors naturally occurring mutations that are associated with drug resistance to protease\(^6\) or reverse transcriptase inhibitors\(^9\).

A reliable and quantitative PCR-based method that is not restricted to HIV-1 subtype B isolates is required in therapy research. The increased prevalence of viral subtypes other than B in the western world\(^5,10-13\) requires a change in research focus to enable for the development of diagnostic tools for the detection of non-B HIV-1 subtypes. Here we describe the development of a highly sensitive real time TaqMan assay that quantifies HIV-1 DNA content of infected cells. Since most HIV-1 quantitation assays that are based on gag or pol are subtype-restricted, we developed a simple quantitative PCR assay that can equally detect the major HIV-1 subtypes. Primers were designed to amplify the highly conserved HIV-1 Long Terminal Repeat (LTR) to quantify fully reverse transcribed HIV-1 DNA.

RESULTS

Primers and probe design. The focus of this study was to develop a sensitive detection assay that quantifies HIV-1 DNA of different subtypes and circulating recombinant forms (CRFs) with equal efficiency. The Los Alamos sequence alignment database was used to calculate the Shannon entropy value and to locate the most conserved viral genome sequences among different HIV-1 subtypes and CRFs (Fig. 1A). The most conserved segments were located in the LTR that encodes the
promoter and other transcription regulatory sequences. Three locations with low sequence diversity were identified (Fig. 1A/insert) and selected to design the TaqMan forward (5’P_F) and reverse (3’P_R) primers and the probe (P_pr) (Fig. 1B).

With primers 5’P_F and 3’P_R many forms of HIV-1 DNA were amplified: from the early steps of reverse transcription after primer binding and elongation to the reverse transcribed linear or circular DNA. With The Shannon entropy analysis additional genomic regions, with low sequence diversity, were identified to design primers for a pre-amplification step. The reverse primer 3’P_RP anneals in the untranslated leader sequence downstream of the primer binding site (PBS) and the forward primer 5’P_FP anneals upstream in the U3 region of the LTR (Fig. 1B&C). With these two primers the assay amplifies fully reverse transcribed viral genomes since this region of the viral genome is copied in the last step of the complex reverse transcription process (reviewed in Abbink and Berkhout14).

After reverse transcription the linear HIV-1 DNA integrates into the host’s genome, a process that is mediated by the viral integrase protein15. Alternatively, a proportion of the viral DNA circularizes as 1-LTR or 2-LTR circles that are dead-end products16. We set out to identify viral sequences with low diversity among the HIV-1 subtypes that could be used for the amplification of either 1- or 2-LTR circles. To exclusively amplify 1-LTR circles the forward primer should preferably align in the viral nef gene. Unfortunately, this genomic region is too diverse among the subtypes to design a primer that would amplify variant subtypes equally well. To amplify the 2-LTR circular HIV-1 DNA form, we designed the reverse primer 3’P_RP2 that anneals in the U3 region of the LTR. In combination with the 5’P_FP, 3’P_RP2 amplifies 2-LTR HIV-1 DNA forms (Fig. 1B&E).

To verify the primer sequence similarity to the different HIV-1 subtypes, the primers and probe were compared to every available sequence covering the specific primer or probe alignment region in the Los Alamos database (Table 1). The analysis shows

**Fig. 1. Primers and probe design. A:** The Shannon entropy values were calculated from 417 full length HIV-1 genome sequences, including subtype A, B, C, D and CRF_01(AE), available from LANL HIV Sequence Database. The X axis indicates the relative nucleotide position of the aligned sequence set. The Y axis shows the Shannon entropy value that was calculated for each nucleotide position and represents the sequence diversity among the aligned viral isolates. The insert circle shows the three spots in the LTR region with the lowest frequency of diversity. **B:** Schematic representation of the HIV-1 genome with the area where the primers and probe anneal (between the grey arrows). The numbers accompanying the arrows represent the nucleotide positions corresponding to the HxB2CG reference sequence. Primers 5’P_F, 3’P_R and the probe P_pr are used in the quantitative TaqMan assay. Primers 5’P_FP and 3’P_RP are used in the pre-amplification to amplify the total reverse transcribed viral DNA content. The primers 5’P_FP and 3’P_RP2 are used in the pre-amplification to amplify 2-LTR circles. **C:** Schematic representation of the primer alignment used for the quantitation of total HIV-1 DNA content. **D:** Schematic representation of the primer alignment in combination with Alu primers for the quantitation of integrated HIV-1 provirus. **E:** Schematic representation of the primer alignment for 1-LTR circles as part of the total HIV-1 DNA content and for the quantitation of circular 2-LTR HIV-1 DNA content.
A

B

C: Linear HIV-1 DNA

D: Integrated HIV-1 provirus

E: 1-LTR and 2-LTR circles
that 78, 86 and 91% of the available sequences from all isolates, that includes group M, N, O and P, are homologous to the TaqMan primers 5'P_f, 3'P_r and the probe P_pr, respectively. There is a single exception: CRF_02(AG) has an insertion in the sequence of primer 3'P_r. The alignment region for pre-amplification primers 5'P_f and 3'P_r is fully conserved in 93% and 94% of the viral isolates, respectively.

**PCR cycle conditions.** The optimal PCR cycle number for the pre-amplification step was identified using linearized TOPO plasmid DNA with nucleotides 1-797 from subtype B, which includes the LTR. A 10 cycle pre-amplification step improved slightly the assay sensitivity when compared with no pre-amplification, whereas 15 cycles gave a more reliable linear range between $2 \times 10^1$ - $2 \times 10^7$ copies per reaction (Fig. 2A). Without pre-amplification the lowest HIV DNA input was not detected consistently, whereas with 20 pre-amplification cycles the highest quantity of input HIV-1 DNA was not within the linear range. We continued using the 15 cycle pre-amplification step that increases the sensitivity yet maintains a broad linear range (Slope= -3.06 ± 0.07, Pearson P<0.0001 and $r^2$=0.9967). The assay was performed 12 times using a full length molecular clone of the HxB2 subtype B isolate with implementation of the 15 cycle pre-amplification step (Fig. 2B). Given that the $2 \times 10^1$ - $2 \times 10^7$ linear range was highly reproducible with subtype B (Slope= -3.14 ± 0.09, Pearson P<0.0001 and $r^2$=0.9960), the next step was to test the other HIV-1 subtypes.

**Detection of different HIV-1 subtypes.** For further development and validation of the assay a panel of HIV-1 subtypes (the isolates: UG273, DJ258 (subtype A), US1, US2, HxB2 (subtype B), UG268, ETH2220, SE364 (subtype C), UG270 (subtype D) and

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Table 1.

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<th>5'P_fp</th>
<th>P_pr</th>
<th>3'P_rp</th>
<th>3'P_r</th>
<th>3'P_rp2</th>
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<tr>
<td>CRF_02 (AG)</td>
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<td><strong>RECOMBINANTS</strong>(^2)</td>
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<td>94.3</td>
<td>96.2</td>
<td>85.7</td>
<td>92.5</td>
</tr>
</tbody>
</table>

\(^1\)The analysis of the section “all isolates” include all available sequences from group M, N, O and P. \(^2\)The recombinants include all available CRFs sequences except CRF_01(AE) and CRF_02(AG).
CM235(CRF_01/subtype AE)) were tested. Serial dilutions of the DNA from each isolate were quantified using the designed primers described above. The assay maintained its sensitivity for all isolates tested (Pearson P<0.0001 and $r^2$ ranging from 0.9617 to 0.9916) (Fig. 3). The linear range remained accurate between $2\times10^1$ and $2\times10^7$ copies per reaction. These results indicate that the assay reliably measured samples with low viral DNA content and detected viral isolates from the major subtypes equally well.

**Influence of cellular genomic DNA.** To address whether cellular background DNA affects the detection of serial dilutions of HxB2 HIV-1 DNA, the assay was performed in

**Fig. 3. Detection of different HIV-1 subtypes:** Quantitation of HIV-1 DNA from subtypes A, B, C, D and AE (CRF-01) using a 15 cycle pre-amplification step prior to the TaqMan assay.
the presence of chromosomal DNA (equivalent to $2.5 \times 10^5$ SupT1 cells). Dead cells, cell debris and other contaminants were removed by sorting the uninfected SupT1 T cells with flow cytometry and the total nucleic acids were isolated utilizing the Boom method\textsuperscript{17}. The HIV-1 DNA copy number was quantified with and without cellular DNA of uninfected SupT1 T cells. The results measured with chromosomal DNA were correlated to those obtained without (1/slope =1.01, Pearson P<0.0001 and $r^2=0.9685$) (Fig. 4) and demonstrate that the presence of chromosomal DNA does not decrease the sensitivity of the assay.

**Quantitation of HIV-1 DNA integrated in T cell lines.** To validate the assay for detection of integrated provirus, SupT1 T cell lines containing an integrated doxycycline (dox)-dependent HIV-rtTA\textsuperscript{**} provirus were used\textsuperscript{18}. These cell lines contain latently integrated provirus when dox is absent. However, if dox is added, these cells start to produce viral particles. Two different cell lines were used, one carrying a single copy of integrated HIV-1 DNA (named SupT1-1) and the other carrying 14 copies (SupT1-14) as determined by fluorescent in situ hybridization (FISH) (Fig. 5A and data not shown). The SupT1-1 and SupT1-14 cells were cultured with dox, harvested and fixed in 3.7% paraformaldehyde. Dead cells and cell debris were removed by sorting the cells with flow cytometry and DNA was subsequently released by Proteinase K treatment. The assay confirmed that the SupT1-1 cells carry a single HIV-1 copy while SupT1-14 cells contain 14 copies per cell (Fig. 5B).

To verify that the assay is not affected by the production of new viral particles the SupT1-1 and SupT1-14 cells were cultured in the presence of dox. The fusion inhibitor T1249 was added to prevent subsequent rounds of infection. Additionally, the samples were subjected to either RNAse or DNase treatment prior to assay. RNAse treatment had no effect while DNase abrogated the signal, demonstrating that HIV-1 DNA measurement is not influenced by the presence of viral RNA (Fig. 5B).

To verify the quantitation of cell numbers by flow cytometry the cell numbers were also quantified with a commercial quantitative TaqMan assay that measures the β-actin house keeping gene. The HIV-1 DNA copy number per $10^5$ cells was
correlated with the amount of cells either quantified by flow cytometry or by β-actin quantitative PCR (qPCR). The results obtained with the two methods correlated strongly (slope=1.22, Pearson P<0.0001 and $r^2=0.9804$) (Fig. 5C). Additionally, the influence of 3.7% paraformaldehyde was tested. Prior to Proteinase K treatment, the SupT1-1 cells were subjected to fixation or not. The results show that fixation of the cells with 3.7% paraformaldehyde did not influence the assay (Fig. 5D).

Next, serial dilutions of the SupT1-1 HIV-1 DNA content were measured in the presence of chromosomal DNA. HIV-1 DNA was isolated with the Proteinase K method and each sample was supplemented with chromosomal DNA equivalent to 6×10^4 uninfected SupT1 cells. The HIV-1 DNA copy number from SupT1-1 cells without cellular DNA correlated to the measured HIV-1 DNA copy numbers with chromosomal DNA (slope=1.10, Pearson P=0.0105 and $r^2=0.98$) (Fig. 5E). This

\[ \text{SupT1-1} \]

\[ \text{SupT1-14} \]

\[ 1 \]

\[ 2 \]

\[ 3 \]

\[ 4 \]

\[ \text{Log}_{10} \text{HIV-1 DNA copies/10^6 cells} \]

\[ \text{SupT1-1} \]

\[ \text{SupT1-14} \]

\[ 1 \]

\[ 2 \]

\[ 3 \]

\[ 4 \]

\[ \text{Log}_{10} \text{HIV-1 DNA copies} \]

\[ \text{Log}_{10} \text{HIV-1 DNA copies} \]

\[ 1 \]

\[ 2 \]

\[ 3 \]

\[ 4 \]

\[ 5 \]

\[ r^2=0.9792 \]

Pearson P=0.0105

\[ 6 \]

\[ 7 \]

\[ 8 \]

\[ \text{Log}_{10} \text{HIV-1 DNA copies} \]

\[ \text{Log}_{10} \text{HIV-1 DNA copies} \]

\[ 1 \]

\[ 2 \]

\[ 3 \]

\[ 4 \]

\[ 5 \]

\[ r^2=0.9792 \]

Pearson P=0.0105

\[ 6 \]

\[ 7 \]

\[ 8 \]

Fig. 5. Quantitation of integrated proviral HIV-1 DNA in SupT1 T cell lines. A: Visualization of integrated HIV-1 DNA in the SupT1-1 T cell line by fluorescent in situ hybridization (FISH), demonstrating that these cells carry a single copy of viral DNA. The arrow indicates the two dots corresponding to the single integrated HIV-1 genome in a dividing cell. B: Quantitation of the HIV-1 DNA in 10^5 SupT1-1 (grey bars) or SupT1-14 (black bars) cells 1: without pre-amplification, 2: 15 cycles of pre-amplification, 3: in the presence of RNase, 4: in the presence of DNase. C: Correlation of the HIV-1 DNA copy numbers and the quantity of SupT1-1 cells. Cell numbers (indicated next to the linear regression curve) were quantified either by flow cytometry or by β-actin TaqMan quantitation. D: Influence of paraformaldehyde fixation on the HIV-1 DNA quantitation in SupT1-1 cells. E: Correlation of HIV-1 DNA quantified in SupT1-1 cells that were supplemented or not with chromosomal DNA equivalent to 6×10^4 uninfected SupT1 T cells.
demonstrates that the HIV-1 DNA measurement is not influenced by the presence of large amounts of chromosomal DNA.

**HIV-1 DNA detection within infected cells and in clinical samples.** The assay was further evaluated for the capacity to quantify HIV-1 DNA copy numbers in primary cells as well as in SupT1 cells infected with HIV-1. PHA-activated CD4+ T cells, immature and mature monocyte-derived dendritic cells (iMDDC and mMDDC) and SupT1 T cells were infected with the HIV-1 strain HxB2 (7.500 TCID50). The cells (10^5) were harvested 2 days after infection and subjected to our viral DNA quantitation assay. Pre-amplification steps for fully reverse transcribed viral genomes (tot-LTR) and 2-LTR circles were performed and the samples were subsequently analyzed in the TaqMan DNA assay. As expected, the fully reverse transcribed HIV-1 DNA copy number per cell, shown as tot-LTR values, varied between the different cell types (Fig. 6A). The SupT1 T cell line showed the highest viral DNA load followed by the CD4+ T cells (0.5 log, lower DNA levels compared to SupT1 T cells). The mMDDCs and iMDDCs showed a similar DNA load, but 0.8 log lower compared to the CD4+ T cells. iMDDCs treated with the fusion inhibitor T1249 served as a negative control to demonstrate that the assay quantifies only post-entry products. Although the 2-LTR copy numbers were three orders of magnitude lower than the tot-LTR copy numbers, the quantified 2-LTR DNA correlated strongly with the measured tot-LTR for each cell type, (Pearson P=0.0006 and r^2=0.9875) (Fig. 6B). The assay was further evaluated utilizing clinical material isolated from 8 recently infected HIV-1 positive individuals. HIV-1 DNA copy-numbers (tot-LTR) were measured in the monocyte fractions and compared with HIV-1 plasma viral loads (Fig. 6C). For each patient HIV-1 DNA could be detected and quantified in the monocyte fractions with up to 1 log variation observed in copy number between individuals. It was also observed that HIV-1 DNA copy-numbers associated with plasma RNA loads.

**DISCUSSION**

The HIV-1 field has benefited immensely from subtype B orientated studies but it has become apparent that new tools are required for research in areas where non-B subtypes are circulating. Particularly in sub-Saharan Africa where many variant subtypes are co-circulating but also within Europe and North America where the number of non-B infections increases due to immigration and tourism19. In addition, HIV-1 is still evolving and the prevalence of recombinant forms is ever increasing20,21. There are multiple quantitative HIV-1 assays that measure viral RNA with high efficiency22 and include the detection of non-B subtypes23-26. Assays that detect viral DNA from non-B subtypes have also been described27,28. However, these assays target the gag/pol region of the viral genome, which is more diverse among HIV-1 subtypes in comparison to the LTR region targeted in the assay described here. Based on phylogenetic analysis, this assay can measure many circulating HIV-1 subtypes with equal efficiency with the likely exception of CRF-02(AG). This HIV-1
subtype differs due to the insertion of a single nucleotide in the target region of the TaqMan primer 3’Pr. However, the analysis depicted in Table 1 is based on perfect similarity and subtypes with one or more mismatches could still be detected.

Increased assay sensitivity is a key requirement in studies where small amounts of HIV-1 infected cells are present amidst large numbers of uninfected cells, specifically in clinical studies monitoring the effect of antiretroviral therapy and the study of elite controllers. The designed quantitative HIV-1 DNA assay using the TaqMan DNA amplification technology detects low copy numbers of HIV-1 DNA in the presence of human genomic DNA from uninfected cells. Performing the assay using only the
TaqMan primers 5’P<sub>F</sub> and 3’P<sub>R</sub> doubles the sensitivity since both 5’ and 3’ LTRs are measured. The investigation of cellular reservoirs could be aided by such measurements, particularly when cell populations with low virus susceptibility are studied. However, as this assay detects different HIV-1 subtypes equally well it can not be used for virus subtyping.

HIV-1 reverse transcription is a complex process where many intermediate or unfinished shorter viral DNA forms can be found in infected cells. The TaqMan primers 5’P<sub>F</sub> and 3’P<sub>R</sub> align in both the 5’ and 3’ LTR regions and therefore amplifies variant HIV-1 DNA intermediates. However, the primers used in the pre-amplification step (5’P<sub>FP</sub> and 3’P<sub>Rp</sub>) exclusively amplify fully reverse transcribed DNA (tot-LTR). Detection of completely reverse transcribed HIV-1 DNA does not necessarily mean that the virus will integrate as provirus. However, it does indicate that the cell is infected and can be useful to monitor as HIV-1 DNA levels are associated with therapy outcome<sup>29</sup>. Reverse transcribed HIV-1 DNA is present in non-integrated (linear and circular (1- or 2-LTR circles)) and integrated forms. By using specific primers in the pre-amplification step the 2-LTR circular DNA form can be quantified. Although the exact role of how non-integrated HIV-1 DNA copies can influence pathogenesis is not clear, the 2-LTR circles are of interest since they accumulate and may serve as marker for persistent viral replication in patients on antiretroviral therapy showing undetectable plasma HIV-1 RNA loads<sup>30-33</sup>. The designed primers can contribute to such studies by including the measurement of several HIV-1 subtypes in a single assay.

A downside of the described experimental design is that it scores the total HIV-1 DNA copy number and does not discriminate between integrated and non-integrated DNA. Integrated HIV-1 provirus can be quantified with a primer targeting the human repetitive Alu element in combination with a primer targeting the provirus<sup>34</sup>, although some proviruses will be missed using this approach<sup>18</sup>. The pre-amplification primers 5’P<sub>FP</sub> and 3’P<sub>Rp</sub> can be used in combination with Alu primers for the detection of integrated provirus (Fig. 1D).

A sensitive TaqMan assay has been developed that can measure a single HIV-1 DNA copy per cell and which can detect major HIV-1 subtypes A, B, C, D and AE (CRF_01) with equal efficiency. The primers and probe used in the TaqMan quantitation assay can be used to score HIV-1 DNA copies in a sample that is pre-amplified in three different manners: 1) total reverse transcribed HIV-1 DNA 2) 2-LTR circular and 3) integrated provirus.
MATERIALS AND METHODS

HIV-1 genome analysis. Full length HIV-1 genome sequences, including the LTR, from the Los Alamos database were selected (n=417). Sequences from 17 subtype A, 200 subtype B, 48 subtype C, 8 subtype D, 6 subtype F, 7 subtype G and 131 from CRF-01(AE) viruses were aligned according to the reference sequence alignments. The Shannon entropy was calculated for each nucleotide position from the full length viral genome with DnaSP 5.10.01 software to define the best conserved genomic regions.

HIV-1 isolates and DNA standards. The HIV-1 isolates selected for this study were UG273, DJ258 (subtype A), US1, US2, HxB2-LAI (subtype B), UG268, ETH2220, SE364 (subtype C), UG270 (subtype D) and CM235 (CRF_01/subtype AE). These selected isolates were acquired from a virus panel that was characterized previously. Peripheral blood mononuclear cells (PBMCs) were infected (multiplicity of infection 0.001) with the HIV-1 isolates listed above and nucleic acids were isolated according to the method described by Boom et al. The full length U3RU5 LTR region (corresponding nucleotides [nt] 1-797 of the virus genome according to the HIV-1 HxB2CG [GenBank accession no. K03455] numbering) was amplified using primers MSF13 and L-GAGUNIM2 (Table 2) under standard PCR conditions with AmpliTaq DNA Polymerase (Applied biosystems). The amplicons were cloned into the TOPO-TA plasmid (Invitrogen) and transformed into E. coli Top-10 bacteria. The plasmids were isolated to generate HIV-1 DNA stocks used as subtype prototypes. The plasmids were linearized with the restriction enzyme BamHI (New England Biolabs) and purified using DNA purification columns (Qiagen) according to the manufacturers instructions. The DNA concentration was quantified with the spectrophotometer ND-1000 UV-Vis (NanoDrop technologies) and verified on a 1% agarose gel. The final DNA concentration was standardized to the equivalent of 2×10^9 copies/μl. 10-fold serial dilutions ranging from 2 to 2×10^6 DNA copies/μl were used in the quantitation assay.

Primers and probe. Primers targeting the most conserved region in the LTR, identified by the sequence analysis, were designed (Table 1) using Mfold and Oligo Analyzer software. Two different pre-amplification primer sets were designed to discriminate between different HIV-1 DNA forms. The first primer set (5’P_R and 3’P_R) detects all forms (linear and circular) of fully reverse transcribed HIV-1 DNA (Fig.1). The second primer set (5’P_R and 3’P_R2) is specific for the 2-LTR circular DNA form. The 15 cycle pre-amplification conditions were: 1 min (94°C), 1min (55°C) and 2 min (72°C) using AmpliTaq DNA Polymerase (Applied biosystems). The pre-amplified PCR product was used as input in the TaqMan measurement.

For the quantitative TaqMan reaction primers 5’P_f, 3’P_R were used together with probe P_f. The probe carries a FAM fluorescence label, a TAMPA quencher and a major groove binding (MGB) group for stability. The quantitative TaqMan conditions
were: 2 min (50°C), 10 min (95°C) and 45 cycles of 15 sec (95°C) followed by 1 min (60°C) using the Platinum TaqMan kit (Invitrogen).

**Cells, Cell lines and clinical samples.** PBMCs were isolated from buffy coats by standard Ficoll-Hypaque density centrifugation. CD8⁺ T lymphocytes were depleted from the PBMC pool, using CD8 immunomagnetic beads (Dynal) and the CD4⁺ enriched T lymphocytes were activated with PHA (2 µg/ml) in the presence of rIL-2 (100 U/ml) prior to infection with HIV-1 as described previously. Clinical materials were collected from HIV-1 positive individuals visiting the outpatient clinic of the Academic Medical Center, Amsterdam (under medical ethical review granted to the Amsterdam Cohorts Studies). At time of sample collection, plasma HIV-1 RNA was measured (bDNA 3.0 assay from Bayer Diagnostics, Berkeley, California, USA), and blood CD4⁺ T lymphocyte, CD8⁺ T lymphocyte, B lymphocyte and monocyte counts were determined. Subsequently, the monocyte cell fractions were isolated from PBMCs as previously described. The assay was performed using 2.5x10⁵ cells since this level of cellular DNA input had been shown not to influence the readout. The human T lymphocytic cell line SupT1 (ATCC CRL-1942) was cultured as previously described. The stable SupT1-1 and SupT1-14 cell lines were generated by limiting dilutions after transducing plasmid DNA of a doxycycline (dox)-dependent HIV-rtTA construct into SupT1 cells as previously described.

**Cell fixation, RNase and DNase treatment.** The HIV-1 infected cell lines were fixed with 3.7% paraformaldehyde before they were sorted and counted by cell flow cytometry. All cell fractions used in the study were fixed with 3.7% paraformaldehyde and treated with Proteinase K (0.1 mg/ml Roche) in 10 mM Tris pH8.3 for 1 h at 56°C. Proteinase K was inactivated by a 10 min incubation at 95°C. The released genomic material was used as input for the quantitation of HIV-1 DNA copy numbers. No further DNA purification was required. For RNase treatment the samples were treated with RNase (Sigma-Aldrich) at a final concentration of 10 µg/ml and for DNase treatment with 50 U/ml DNase I (RNase-free, Applied Biosystems).

**Fluorescent in situ hybridization (FISH).** SupT1-1 and SupT1-14 cells were cultured for 24 h before the cells were incubated with a hypotonic solution of 0.75M KCl to

<table>
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<tr>
<th>Primer name</th>
<th>Nucleotide sequence</th>
<th>Direction</th>
<th>HxB2(K03455) nt position</th>
<th>Tm °C:</th>
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</thead>
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<tr>
<td>MSF13</td>
<td>TGGAAGGGTAATTTACTCCAGA</td>
<td>sense</td>
<td>1→23</td>
<td>69</td>
</tr>
<tr>
<td>L-GAGUNIM2</td>
<td>GCACCCCATCTTCTCTCTAGGCTCCGC</td>
<td>antisense</td>
<td>769→797</td>
<td>99</td>
</tr>
<tr>
<td>5’P₂</td>
<td>GCCCTAATAAGCTTGCTTGA</td>
<td>sense</td>
<td>522→543</td>
<td>65</td>
</tr>
<tr>
<td>3’P₁</td>
<td>GGGGCCACTGCTAGAGAT</td>
<td>antisense</td>
<td>625→643</td>
<td>67</td>
</tr>
<tr>
<td>P₁v</td>
<td>GTA[a/r/g]CTAGAGATCCCTCAGA</td>
<td>probe</td>
<td>584→603</td>
<td>&gt;61</td>
</tr>
<tr>
<td>5’P₁v</td>
<td>TAACCTCTAGAGCTGGGTA[a/r]AAGCAGC[+];TGCT</td>
<td>sense</td>
<td>409→440</td>
<td>&gt;95</td>
</tr>
<tr>
<td>3’P₁v</td>
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<td>688→707</td>
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<tr>
<td>3’P₁vR2</td>
<td>GCTGCTT[a/r]TATGCAAGA[a/r]TGCTAGG</td>
<td>antisense</td>
<td>412→436</td>
<td>&gt;69</td>
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</tbody>
</table>
allow swelling of the nuclei. The cell membrane was dissolved in 0.05 % Tween in 0.1 M HCl and the cells were fixed with methanol/acetic acid (3:1) on a glass slide. FISH was performed with a dUTP biotin-labeled full length HIV-1 probe. A secondary avidin-Cy3 labeled antibody was used to visualize the bound probe by confocal microscopy. Uninfected SupT1 cells were used as a negative control.

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We thank Stef Heijnen for performing CA-p24 ELISA and Berend Hooibrink for expert advice on the flow cytometry facility and assistance with the FACS-ARIA cell sorter. Part of the work was supported by the Dutch AIDS Fund (Aids Fonds 2007028 and 2008014).

REFERENCES