A universal real-time PCR assay for the quantification of group-M HIV-1 proviral load

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Quantification of human immunodeficiency virus type-1 (HIV-1) proviral DNA is increasingly used to measure the HIV-1 cellular reservoirs, a helpful marker to evaluate the efficacy of antiretroviral therapeutic regimens in HIV-1–infected individuals. Furthermore, the proviral DNA load represents a specific marker for the early diagnosis of perinatal HIV-1 infection and might be predictive of HIV-1 disease progression independently of plasma HIV-1 RNA levels and CD4$^+$ T-cell counts. The high degree of genetic variability of HIV-1 poses a serious challenge for the design of a universal quantitative assay capable of detecting all the genetic subtypes within the main (M) HIV-1 group with similar efficiency. Here, we describe a highly sensitive real-time PCR protocol that allows for the correct quantification of virtually all group-M HIV-1 strains with a higher degree of accuracy compared with other methods. The protocol involves three stages, namely DNA extraction/lysis, cellular DNA quantification and HIV-1 proviral load assessment. Owing to the robustness of the PCR design, this assay can be performed on crude cellular extracts, and therefore it may be suitable for the routine analysis of clinical samples even in developing countries. An accurate quantification of the HIV-1 proviral load can be achieved within 1 d from blood withdrawal.

INTRODUCTION

Real-time PCR is becoming increasingly popular as a method for the quantitative detection of DNA and RNA viruses. Besides its research applications, an accurate determination of the viral load, whether cell associated or free in biological fluids, can provide critical information in the clinical setting for the diagnosis and staging of acute and chronic viral infections[1–7], as well as for guiding treatment interventions and assessing their efficacy[8–10]. In addition, real-time PCR has been applied for screening purposes to measure the level of contaminating viruses in blood donations or plasma pools for the production of blood derivatives[11–16].

In HIV-1 infection, the determination of the number of cell-associated HIV-1 DNA copies (HIV-1 proviral load) by real-time PCR is employed not only for research purposes[17–22] but also for evaluating the effects of antiretroviral therapy on the viral reservoirs[23–28]. Furthermore, a high HIV-1 proviral DNA load following primary infection has been suggested to be predictive of rapid disease progression independently of plasma viremia and CD4$^+$ T-cell counts[29,30].

Despite the increasing number of studies involving the quantification of cell-associated HIV-1 DNA load, some of the methodologies employed are semiquantitative and require laborious post-amplification analytical procedures[25]; other methods either lack sufficient sensitivity[7,29] or cannot correctly quantify all the HIV-1 M genetic subtypes, as the HIV-1 regions selected for probe and primers design are conserved in less than 70% of the HIV-1 isolates available in the Los Alamos database[7,31].

Real-time quantitative PCR is a state-of-the-art technology that combines the amplification and detection steps of the PCR in a single-tube format[31,32]. The assay measures in real time an increase in fluorescence signal that is proportional to the amount of DNA that is *de novo* synthesized during each PCR cycle. The use of probes labeled with different reporter dyes allows for the detection and quantification of multiple target genes within a single reaction. The initial amount of target DNA determines at which PCR cycle the fluorescent signal rises above a defined threshold of background fluorescence; this cycle is denoted as the threshold cycle (C_t). The normalized fluorescent signal (ΔRn) generated by the hybridized probe is automatically calculated by a computer algorithm that normalizes the reporter emission signals. The algorithm then calculates the C_t at which each PCR reaches the ΔRn threshold value (usually set at 10 times the standard deviation of the baseline signal), which is inversely proportional to the log number of target copies initially present in the test sample. The correlation between the fluorescent signal and the amount of amplified product permits an accurate quantification of target molecules over a wide dynamic range, while retaining the sensitivity and specificity of conventional end-point PCR assays.

Real-time PCR offers several important advantages over conventional quantitative PCR methods by substantially reducing labor and costs because a single PCR run is sufficient to accurately quantify target DNA without any post-amplification steps. Moreover, the absence of post-amplification manipulation steps greatly reduces the risk of inter-sample contamination and eliminates the need for employing radioactive labels or other hazardous reagents. Finally, by virtue of its high-throughput format, this system is well-suited for automatization and use in the routine clinical diagnostic setting. Here, we describe a real-time PCR protocol for the bona fide quantification of the HIV-1 DNA load that can be applied equally well to experimental and clinical settings[20,21,27]. Although a very limited number of variant isolates may still escape accurate quantification, this protocol is able to correctly measure HIV-1 DNA from a wide panel of viral isolates of diverse genetic subtypes[33].

Experimental design

Protocol overview. Quantification of the HIV-1 proviral DNA is increasingly used in the clinical follow-up of HIV-1–infected individuals. However, due to the high degree of genetic variability of HIV-1, the identification of a PCR amplicon that guarantees the same efficiency of amplification with all the genetic subtypes of the main (M) HIV-1 group represents a major challenge to the design of a universal assay[34–36]. We have developed an HIV-1 group M-specific quantitative real-time PCR assay that measures the HIV-1 proviral DNA load with a similar degree of sensitivity and accuracy regardless of the viral genetic subtype[20,21,27,33,37]. The success of this protocol for the quantification of HIV-1 proviral DNA relies on the optimized design of primers and probe, which ensures the correct quantification of virtually all circulating group-M HIV-1 strains. Moreover, this assay is so robust that it can quantify DNA derived from a crude lysate with the same degree of accuracy as with purified DNA.

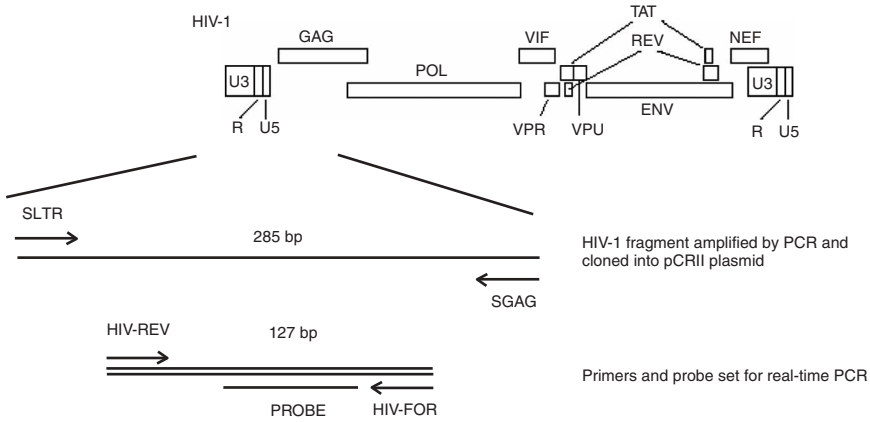

Figure 1 | Schematic representation of the HIV-1 region selected for the development of the HIV-1 proviral DNA real-time PCR assay.

Assay design. As a starting point, we selected a region spanning parts of the HIV-1 LTR and the *gag* gene, which is highly conserved among all circulating group-M HIV-1 subtypes (**Fig. 1**). The selected region is completely conserved in about 91% of the HIV-1 sequences present in the NCBI database (**Table 1**). A first real-time PCR assay (HIV-1 Standard System) was developed by designing two specific primers, designated as HIV-FOR and HIV-REV, and a probe within the selected LTR-*gag* region (**Fig. 2**). To obtain a standard template, DNA was extracted from the T lymphoblastoid cell line 8E5 (ATCC 8993), which contains a single proviral genome of HIV-1, strain LAV. A fragment containing the selected region was amplified by PCR using a pair of primers, SLTR (5′-CTAGCAGTGGCGCCCGAA-3′) and SGAG (5′-CTAGCTCC CTGCTTGCCCA-3′), which are complementary to a region external to the binding sites of the real-time PCR primers. PCR amplification was carried out as described[33]. The 285-bp amplified product was cloned into the pCRII plasmid, and plasmid DNA was expanded, purified and accurately quantified by UV-light spectroscopy. Finally, serial dilutions of the plasmid were prepared (from 10^6 to 10^{-1} copies per reaction) and used to build a reference curve for quantification. The real-time PCR was performed using primers HIV-FOR and HIV-REV as described in the PROCEDURE.

Next, we evaluated the accuracy and sensitivity of the HIV-1 Standard System on a panel of primary HIV-1 isolates of different genetic subtypes, some of which were carrying mutations in either the primers or the probe sequences. For this purpose, we selected 31 primary HIV-1 isolates representing genetic subtypes A–F (ref. 33) and obtained them through the UNAIDS Network for Viral Isolation and Characterization. DNA from each isolate was amplified by PCR with primers SLTR and SGAG, and the amplified products were cloned into the pCRII plasmid. A total of 119 clones was expanded, purified and accurately quantified by UV-light spectroscopy. Each clone was sequenced by Thermo Sequenase cycle sequencing using the Cy5-labeled M13 REV primer (5′-CAGGAAACAGCTATGACC-3′). The sequences obtained were submitted to the Los Alamos database. As illustrated in **Table 2**, analysis of the efficiency of the HIV-1 Standard System on these clones showed a highly efficient and reproducible amplification pattern in the vast majority of the cases, with a decrease in quantification accuracy in only 2 of 119 (1.7%) clones analyzed. These two clones, obtained from two independent subtype-A isolates, presented either an insertion (A) at position 9 of the HIV-REV primer (isolate 9611549-2) or a nucleotide substitution (14C→T) within the same primer (isolate 92UG037-3). To overcome this drawback, although minor, we therefore designed an HIV-1 Modified System containing an alternative reverse primer (9insA) and increased the concentration (1.5 μM) of the reverse primer. The modified assay was able to accurately measure proviral DNA from all the primary HIV-1 isolates tested with similar efficiency regardless of genetic subtype (see below) as well as from clinical samples derived from HIV-1-infected patients[20,21,27].

Controls. In each assay, serial dilutions of the cloned plasmids are used to generate the standard curves as well as to obtain accurately quantified positive controls. Two negative controls are typically included in each plate: a reference negative control, usually DNase/RNase-free water, as a control for the PCR, and an HIV-negative human sample for monitoring the extraction procedure (typically DNA extracted from blood leukocytes of a HIV-seronegative individual and stocked in multiple aliquots at −80 °C) (**Fig. 3**). Both the standard curve and the negative controls are tested in each

TABLE 1 | NCBI database analysis for HIV-1 isolates carrying mismatches within the selected primers and probe sequences.

Mismatches	Number of HIV-1 sequences (%) in the NCBI database		
	HIV-FOR primer	HIV probe	HIV-REV primer
None	4,881 (90.82)	1,924 (99.7)	1,810 (97.52)
3G→A	249 (4.63)		
4A→G	245 (4.55)		
9A→G		5 (0.3)	
9insA			16 (0.86)
13G→A			5 (0.28)
14C→T			25 (1.34)

The numbers of recovered HIV-1 sequences presenting no mismatch or the indicated mismatches are reported. Only mutations with a frequency higher than 0.2% are shown. 9insA: an alanine insertion in position 9 of the HIV REV primer.

experiment. The unknown sample is tested in triplicate to ensure an accurate quantification as well as the highest sensitivity of the assay (equal to 1 viral genome equivalent/reaction). A semilogarithmic plot of log-DNA concentration versus C_t should yield a Pearson correlation coefficient (R^2) greater than 0.97. The amount of DNA present in the unknown sample is calculated by interpolation from the standard curve.

DNA extraction. In standard PCR assays, a critical requirement is that the DNA must be free of contaminants such as hemoglobin, polysaccharides, alcohol or high salt concentrations, as these may interfere with the PCR. In our protocol, we employ two different methods to prepare DNA: extraction with phenol–chloroform or lysis buffer B (see PROCEDURE below). Strikingly, although the second method does not result in a high degree of DNA purification, the robustness of our real-time assays permits to overcome the interference of potential contaminants like detergents, salts, proteins or even cellular membrane fragments. The great advantage of the crude lysis method is that quantification is obtained directly from the cellular extract without any intermediate step of DNA purification that might lead to variable and unpredictable levels of DNA loss.

Cell number assessment and normalization. An essential requirement for a bona fide absolute quantification of proviral HIV-1 DNA in clinical samples is the assessment of the number of human cells present in the samples before the DNA extraction step. To normalize each clinical sample for human genomic DNA content (i.e., number of cells), we have designed and developed an additional real-time PCR assay that must be performed on each sample to measure the copy number of a single-copy human gene, CCR5, located on chromosome 3. The CCR5 amplicon was chosen in a region of the CCR5 gene (nt. 3,175–3,255) that is highly conserved among all human ethnic groups. The CCR5 assay permits to determine the number of human cells initially present in each clinical sample with high degree of accuracy. Furthermore, the measurement of CCR5 copy numbers serves as internal control for PCR artifacts (i.e., the presence of inhibitors of the amplification reaction). It should be noted that the quantification of the CCR5 gene copy number has to be performed separately from the HIV-1 system because these two assays have not yet been standardized in a multiplex format (single-tube reaction).

Primer design. There are numerous software packages available for PCR primer design, some freely available and some commercial. When designing a new assay, the major goal is achieving the highest possible efficiency and sensitivity, avoiding the formation of primer dimers. To maximize specificity, DNA primers should be between 15 and 25 bases long, with a G/C content of approximately 50%. Primers that may form secondary structures (i.e., inverted repeats) or with sequence complementarity at the 3′ ends, which could result in dimer formation, should be avoided. Owing to competition between intermolecular (primer-template, probe-target) hybridization and intramolecular hybridization, inverse repeats can cause inefficient priming and probing of the target sequence. The PCR can completely fail because of the formation of stable hairpins at the binding region, or inside the amplicon in general. Primer dimers have a negative ΔG value, so primers should be chosen with a value that is not more negative than -10 kcal mol^{-1}. The 3′ terminal position is essential for the control of mispriming during the PCR. Primers with one or more G or C residues at the 3′ end will have increased binding efficiency due to the stronger hydrogen bonding of G/C residues. The presence of a G or C at the 3′ end also helps to improve the efficiency of priming by minimizing the mispriming phenomena that might occur. However, an excess of G and C at the primer 3′ that may cause clamping should be avoided because ambiguous binding of oligonucleotides to the target site could result in misprimed elongation ('slippage effect'). A reasonable compromise is to include no more than 2 or 3 G/C among the first 5 bases at the primer 3′ end. Similarly, long repeats of a single base (i.e., more than 3–4 identical bases in a row, especially G or C) should be avoided, as homopolymeric runs can also cause the 'slippage effect'. In general, the primer concentration for probe-based assays is 200–400 nM.

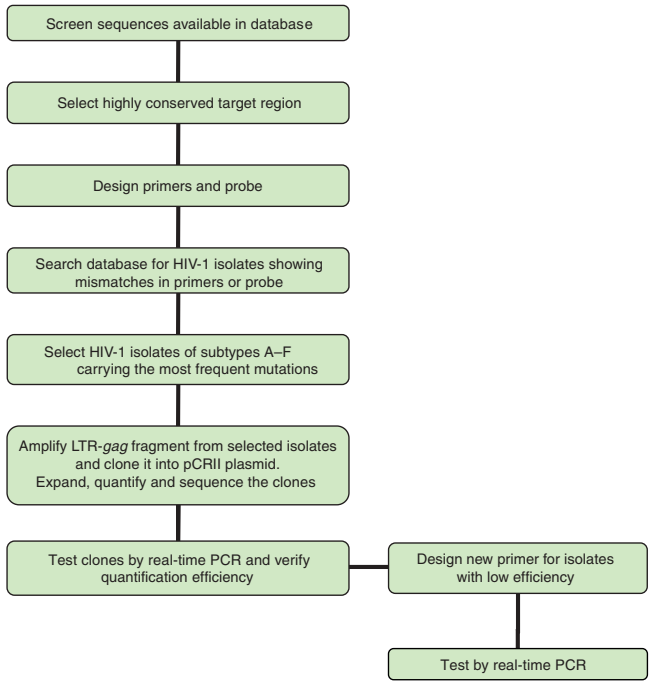

Figure 2 | Flow chart illustrating the various steps toward the setup of the HIV-1 proviral DNA quantification assay.

TABLE 2 | Evaluation of the accuracy of the HIV-1 quantification system using isolates belonging to various HIV-1 subtypes.

Isolate (subtype)	Mismatches			Accuracy[a]
	HIV-FOR	HIV probe	HIV-REV	
92RW009-2 (A)	3G→A			1.0
92TH014-1 (B)	3G→A			1.0
953971-1 (D)	4A→G			1.0
92BR025-12 (C)	3G→A		13G→A	0.9
92BR016-3 (E)		9A→G		0.8
9611549-2 (A)			9insA	0.3
92UG037-3 (A)			14C→T	0.2

[a]Accuracy was calculated by dividing the number of copies measured with the standard primer/probe combinations by the number of copies measured with the fully matched primer and probe combination specific for each mutation. Accuracy did not vary regardless of the starting amount of template used (10^5, 10^3 or 10^2 genome equivalents per reaction).

Probe design. qPCR assays using hydrolysis probes (such as TaqMan) are usually carried out as two-step reactions, with a denaturation step followed by a combined annealing/polymerization step during which fluorescence emission is measured. Most detection systems rely upon binding of the probe to the template

before hybridization of the primers. For this reason, the probe T_m should be approximately 10 °C higher than the T_m of the primers, usually within the 68–70 °C range. The probe should be designed with the 5′ end as close as possible to the 3′ end of one of the primers, but without overlap. This ensures rapid cleavage by the polymerase. The probe should be no more than 30 nucleotides in length to maximize quenching, with a G/C content of approximately 50%. If the target sequence is AT rich, analogs such as minor groove binders (MGB; Applied Biosystems) should be incorporated. MGBs are dsDNA-binding agents that are attached to the 3′ end of TaqMan probes and stabilize the hybridization reaction. This allows for the design of shorter probes, which increases design flexibility. As guanine (G) can naturally quench fluorescence, it should not be present as the 5′ base. When placed in this position, G would continue to quench the reporter dye after cleavage, resulting in reduced fluorescence values (DR) and, therefore, reduced sensitivity. Any runs of four or more identical nucleotides, especially G, should be avoided because they could affect the secondary structure of the probe and reduce hybridization efficiency.

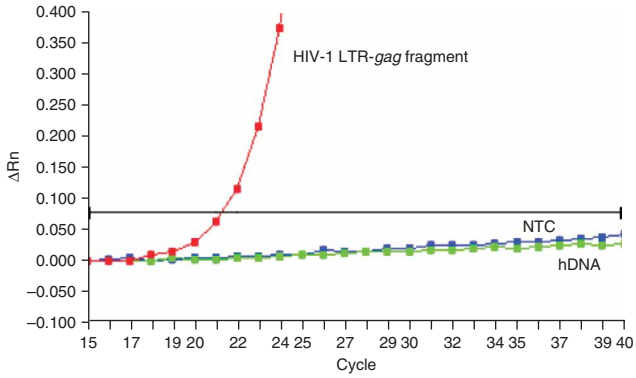

Figure 3 | Amplification plots obtained with the HIV-1 plasmid control (shown in red), uninfected human genomic DNA (hDNA; green) and a no-template control (NTC; blue). The horizontal black line denotes the threshold of background fluorescence. Three different controls should be included in each experiment: the HIV-1 template, a negative control for the PCR (without template) and a negative control for sample extraction (human genomic DNA). The fluorescence emission curves for the negative controls must remain entirely below the threshold line.

MATERIALS

REAGENTS

- KCl (JT Baker, 0208)
- HCl (Merck, K 34071617)
- $MgCl_2$ (AppliChem, A4425)
- Tween-20 (AppliChem, A1389)
- Nonidet-P40 (AppliChem, A1694)
- Proteinase K (BDH, 390973P)
- Polyoxyethylene 10 Lauryl ether (Sigma, P-9769)
- Phenol (BDH, 43675 4G)
- Chloroform (BDH, 100776B)
- Isoamyl alcohol (AppliChem, A0875)
- Isopropyl alcohol (BDH, 102246L)
- NaCl (BDH, 443827W)
- Glycogen (AppliChem, A2168)
- Ethanol (Merck, K37727483)
- Tris (BDH, 194855RT)
- EDTA (AppliChem, A1104)
- Human Genomic DNA from healthy donor
- Viral DNA from T lymphoblastoid cell line 8E5 (ATCC, 8993)
- DNase/RNase free water (Bioline, BIO-38030)
- TOPO TA Cloning kit (with pCR2.1) (Invitrogen, KNM4500-40)
- QIAprep Spin Miniprep kit (Qiagen, 27106) ▲ **CRITICAL** This kit can be used to purify plasmid DNA from bacterial cultures. For preparation of plasmid DNA, other suitable kits can be used; other purification methods that generate high-quality DNA are also acceptable.
- Primers 100 µM (see **Table 3**) (PRIMM, Biotech's Oligonucleotide Synthesis Services). Primers can be synthesized by most vendors
- VIC-TAMRA-labeled and FAM-MGB-labeled probes (see **Table 3**) (Applied Biosystems) ▲ **CRITICAL** Primers and probes should be ordered at different times, requesting the manufacturer to avoid lyophilization, as this can result in cross-contamination. Stock solutions should be prepared with DNase/RNase-free water and aliquoted to avoid whole batch-contamination and repeated freeze/thawing cycles. Probes should be protected from light and stored at −20 °C, either as lyophilized salt or as a 5 µM forward and reverse PCR primers, at 10 µM or as a stock at 10× working concentration solution. Long-term storage of stock solutions is variable, ranging from 6 months to several years.
- Absolute QPCR ROX master mix (ABgene, AB1138/B)

EQUIPMENT

- Microcentrifuge
- Thermomixer
- Primer Express software (Applied Biosystems)
- Oligo 6 software (Molecular Biology Insights)
- Nanodrop ND-1000 spectrofotometer
- ABI 7700 Real-Time PCR System thermocycler (Applied Biosystems) ▲ **CRITICAL** Other real-time PCR thermocyclers may also be used.
- Thermofast 96 semi-skirted plates (ABgene, AB0900)
- Ultraclear cup strips (ABgene, AB0866)

REAGENT SETUP

Florence buffer 100 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 0.5% (vol/vol) Tween-20 and 0.5% (vol/vol) of N-P40. It can be stored at room temperature (RT; ~25 °C) for at least 6 months.

Lysis buffer (B) Tris-HCl, pH 8, 10mM, and Polyoxyethylen 10 Lauryl-ether 0.1% (vol/vol). It can be stored at RT for at least 6 months.

AE buffer Tris 5mM; EDTA 0.5mM.

PROCEDURE

Lysis of samples for DNA extraction

1| The lysis procedure can be performed using option A (for blood cells and tissues) or option B (for purified peripheral blood mononuclear cells (PBMC) or cell lines).

(A) Lysis of biological samples (blood cells and tissues)

● **TIMING 30 min**

(i) Pipette 450 µl of Florence Buffer and 50 µl of Proteinase K (0.1 mg ml^{-1}) into each tube containing a dry pellet of cells (10^6–10^7).

(ii) Vortex and incubate tubes at 56 °C overnight.

(iii) Lysates from blood cells and tissues must now be subjected to genomic DNA extraction as described in Steps 2–16.

TABLE 3 | Primers and probe sequences for the CCR5 and HIV-1 real-time PCR systems.

Assay	Sequence 5′→3′
CCR5	
Forward	ATGATTCCTGGGAGAGACGC
Probe	VIC-AACACAGCCACCACCCAAGTGATCA
Reverse	AGCCAGGACGGTCACCTT
HIV-1	
Forward	TACTGACGCTCTCGCACC
Probe	FAM-CTCTCTCCTTCTAGCCTC
Reverse	TCTCGACGCAGGACTCG
Reverse 9insA	TCTCGACGCAAGGACTCG

(B) Lysis of biological samples (purified PBMC or cell lines) ● **TIMING 2 h 30 min**

(i) Suspend dry pellets of cells (between 1 and 2 × 10^6 cells) in 90 µl of lysis buffer and add 10 µl of Proteinase K (0.1 mg ml^{-1}).

(ii) Incubate at 65 °C for 2 h.

(iii) Incubate at 95 °C for 15 min.

(iv) Lysates from purified PBMCs or cell lines may be further processed for DNA extraction by following Steps 2–16; however, these lysates can alternatively be directly assessed by real-time PCR by proceeding directly to Step 17.

■ **PAUSE POINT** The protocol can be stopped at this step. Samples can be stored at 4 °C for 1 week or at −20 °C for several months before testing by the real-time PCR protocols.

Extraction of genomic DNA ● **TIMING 5 h or 2 d**

2| Prepare a PCIAA (phenol + cloroform + isoamyl alcohol 25:24:1) solution and dispense 0.5 ml in 1.5-ml Eppendorf tube.

3| Add 0.5 ml of lysate sample (from Step 1A(iii) or 1B(iv)), vortex for 20 s and centrifuge the mixture at 15,000*g* for 10 min at RT.

▲ **CRITICAL STEP** Two layers are formed: the upper aqueous phase containing DNA and the lower contaning cellular debris and proteins.

4| Remove the upper layer by pipetting (discarding the lower layer as waste) and transfer into a new 1.5-ml Eppendorf tube with 0.5 ml of PCIAA.

5| Vortex for 20 s and centrifuge at 15,000*g* for 10 min at RT.

6| Remove the upper layer and transfer it into a new 1.5-ml Eppendorf tube with 0.5 ml of CIAA (cloroform + isoamyl alcohol 24:1) solution.

7| Vortex for 20 s and centrifuge at 15,000*g* for 10 min at RT.

8| Transfer the supernatant into a new 1.5-ml Eppendorf tube with 445 µl of cold (stored at –20 °C) isopropanol + 50 µl of NaCl 3 M + 5 µl of glycogen (200 ng $µl^{-1}$).

9| Invert the tube 10 times (do not vortex).

10| Store for a minimum of 2 h at −20 °C.

■ **PAUSE POINT** Protocol can be stopped at this step. Samples can be left overnight at −20 °C or −80 °C.

11| Centrifuge samples at 15,000*g* for 1 h at 4 °C.

12| Discard the supernatant and add 0.5 ml of cold (stored at −20 °C) ethanol 70%.

13| Centrifuge at 18,000*g* for 15 min at 4 °C.

14| Discard ethanol by pipetting (remove all traces and drops) and dry at RT for not less than 10 min.

15| Add 100 µl of Buffer AE to resuspend DNA.

16| Incubate at 56 °C for 30 min.

▲ **CRITICAL STEP** If alternative methods for lysis/extraction have to be used, they should be tested with plasmid dilutions before running the test samples.

■ **PAUSE POINT** The protocol can be stopped at this step. Samples can be stored at 4 °C for up to 1 month before testing for HIV-1 DNA content.

Preparation of standard curve ● **TIMING 2 h**

17| Measure plasmid DNA concentration by spectrophotometric analysis.

18| Dilute plasmid stock to a concentration of 50 ng $µl^{-1}$ (corresponding to 10^{10} copies $µl^{-1}$).

▲ **CRITICAL STEP** For a vector of ~3 Kb, the double-stranded DNA concentration of 50 µg ml^{-1} corresponds to 1.09 × 10^{13} molecules ml^{-1}.

19| Prepare a set of serial tenfold dilutions from 10^9 copies $µl^{-1}$ to 10^{-2} copies $µl^{-1}$ as described in **Box 1**.

BOX 1 | PREPARATION OF TENFOLD SERIAL DILUTIONS OF PLASMID

1. Dispense 900 µl of AE buffer into twelve 1.5-ml Eppendorf tubes.
2. Transfer 100 µl of the diluted plasmid stock (from Step 18 of PROCEDURE) into a 1.5-ml Eppendorf tube with AE buffer.
3. Invert 30 times (do not vortex).
4. Incubate at 56 °C for 5 min.
5. Keep on wet ice for 1 min.
6. For each subsequent dilution, transfer 100 µl of the previous dilution into a new 1.5-ml Eppendorf tube containing 900 µl of AE buffer and then repeat steps 3–6.

▲ **CRITICAL STEP** To avoid the risk of contamination, dispense first AE buffer into each 1.5-ml Eppendorf tube; prepare one dilution point at a time and remove it immediately from the work area. Wear a clean pair of gloves for each dilution.

■ **PAUSE POINT** Plasmid dilutions can to be stored at 4 °C for up to 1 week until the necessary controls on repeatability and reproducibility of the standard curve are performed. Each dilution can be divided in small aliquots (50 µl) and stored at −20 or −80 °C for several months.

Preparation and execution of the real-time PCR assays ● **TIMING 3 h**

20| In a clean reservoir, assemble the PCR master mix as described in **Table 4**. Prepare a single reaction mix for *n* numbers of samples to be tested. Two negative controls (one without DNA and one with HIV-negative DNA) and a series of dilutions of reference DNA (CCR5 or HIV plasmids) should always be tested in parallel to the test samples.

▲ **CRITICAL STEP** For CCR5 quantification, use the CCR5 primers and probe; for the HIV-1 Standard System, use primers HIV-FOR and HIV-REV at 300 nM; for the HIV-1 Modified System, the final concentrations are HIV-FOR, 300 nM; HIV-REV, 1,500 nM; HIV-9insA, 300 nM (all the sequences are reported in **Table 3**).

21| Pipette 15 µl of the above mix (from Step 20) into each well of a 96-well microplate.

▲ **CRITICAL STEP** We recommend testing samples in triplicate for accurate quantification. To avoid the risk of sample contamination, load all replicates vertically, and when each strip is completed, seal it immediately using flat caps. Load the reference standards only after having loaded and sealed the test samples.

22| Spin the microplates if necessary to remove drops and air bubbles.

▲ **CRITICAL STEP** We recommend the use of flat caps and not plastic foil covers to prevent cross-contamination among samples and/or plasmid standards.

23| Run the real-time PCR using the following thermocycle program: 95 °C for 15 min (activation of Ampli*Taq* Enzyme in the *Taq*man MasterMix); 40 cycles of 95 °C for 15 s (denaturing template), 60 °C for 1 min (primer annealing and template extension).

24| Plot the C_t versus log-concentration for the serial dilutions of the reference DNA to generate a standard curve. The Excel software can be used to produce this plot (using the formula $y = ax + b$, where a defines the slope and b the intercept of the log concentration) and calculate the Pearson correlation coefficient (R^2), which defines how well the standard fits to a straight line.

25| Calculate sample concentration from this plot, knowing the test sample's C_t.

? TROUBLESHOOTING

● **TIMING**

Step 1A, lysis procedure of blood cells and tissues: 30 min (+ incubation time)
Step 1B, lysis procedure of purified PBMC or cell lines: 2 h 30 min
Steps 2–16, extraction procedure (after 1A): 5 h or 2 d (optional)
Steps 17–19, preparation of standard curve: 2 h
Steps 20–25, real-time PCR assay and data analysis: 3 h
Note: A complete quantification of the HIV-1 proviral DNA load employing Step 1B lysis procedure and Steps 20–25 for the determination of the sample's cell number (CCR5 assay) and HIV-1 load (HIV-1 Standard or Modified Assays) requires one full day of work.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 5**.

TABLE 4 | PCR mix setup.

Reagents	Amount per well
Master mix	12.5 µl
Forward primer (10 µM)	0.75 µl
Reverse primer (10 µM)	0.75 µl
Probe (5 µM)	1.0 µl

TABLE 5 | Troubleshooting table.

Problem	Possible reason	Solution
Undetectable signal	Primer or probe are missing from the PCR mix	Repeat the run
	Primers or probe are degraded	Resynthesize primers or probe
	Sample is degraded	Restart extraction with new sample aliquot
	Hot-start DNA polymerase was not activated	Ensure that the appropriate initial incubation at 95 °C was performed
	Template concentration is too high	Dilute template and repeat the run
Replicates show high variability	Samples are not warm enough	Before loading, maintain sample for 10 min at 95 °C and for 2 min on ice
	Poor pipetting	Control pipette calibration
	Presence of PCR inhibitors	Reextract sample with phenol–chloroform
C_t recorded in blank NTC	Reagent contamination	Use fresh reagents
	Environmental contamination	Use detergents that hydrolyze and degrade DNA to remove all residual DNA from pipettes, centrifuges and hood
	Instrument contamination	Use exclusively filter pipette tips
High background	Malfunctioning fluorophore in probe (reporter or quencher)	Resynthesize probe

A complete lack of HIV-1 detection or an inaccurate quantification of proviral HIV-1 DNA (poor reproducibility of replicates) is often due to the low quality or quantitative inadequacy of the DNA within the test sample. Therefore, it is strongly recommended to run the CCR5 assay before performing the HIV-1 quantification, as this assay can provide two critical pieces of information: first, the amplification of the CCR5 gene permits the verification in advance of the quality of the material under examination (e.g., the presence of PCR inhibitors); second, the quantification of the CCR5 products permits the assessment of the number of cells present in the sample and, as a consequence, the sensitivity of the HIV-1 proviral load quantification. If the sample contains more then 150,000 cells per reaction, a predilution step is recommended, especially when the crude lysate protocol is used. If the sample contains less than 1,000 cells per reaction, a concentration step is recommended because the level of sensitivity of the test ($\geq$1,000 HIV-1 genome equivalent per 1,000,000 cells) is not sufficient.

ANTICIPATED RESULTS

Analysis of the data from the standard curve can provide substantial information about the assay. For this reason, assays should be initially validated on serial dilutions of high-quality plasmid DNA. The standard curve is constructed from a measure of C_t (y axis) against log template quantity (x axis); the C_t of unknown samples can then be compared to this curve

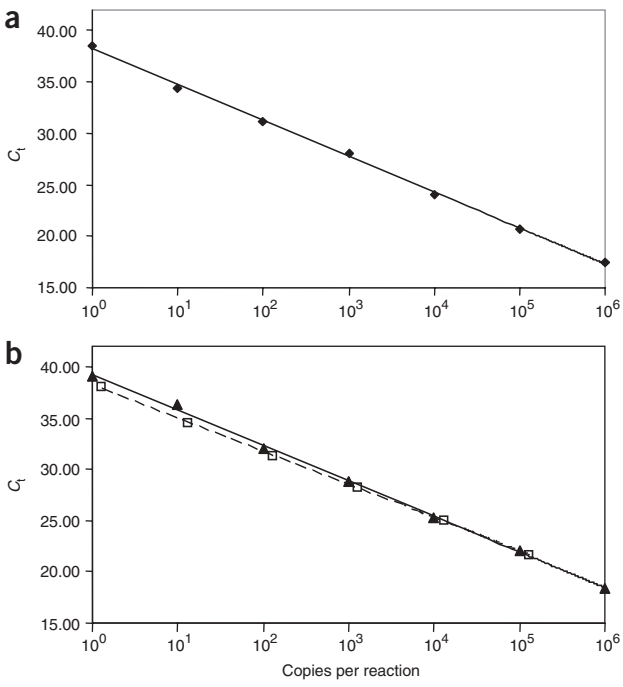

Figure 4 | Standard curves for the CCR5 and the HIV-1 real-time PCR assays. (**a**) CCR5 (◆ $y = -3.48 \log(x) + 38.197$; $R^2 = 0.999$). (**b**) HIV-1 (▲ $y = -3.46 \log(x) + 39.261$ $R^2 = 0.999$). The addition of human genomic DNA up to a concentration of 1 µg per reaction failed to interfere with HIV-1 quantification (□ $y = -3.25 \log(x) + 38.239$; $R^2 = 0.999$).

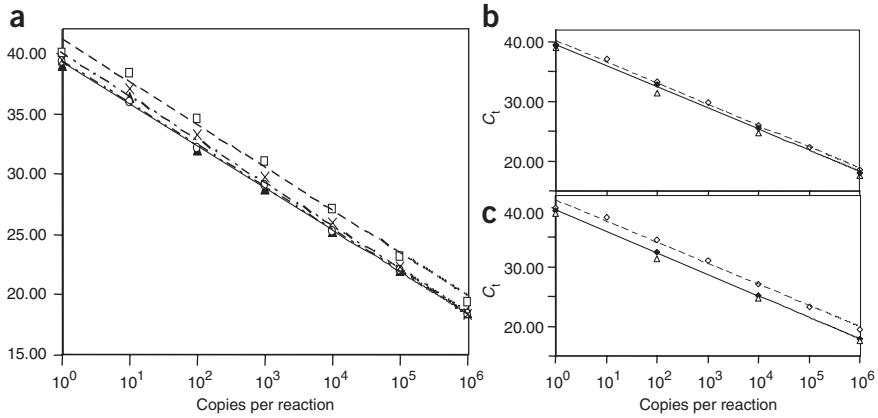

Figure 5 | Effects of HIV-1 genetic variability on HIV-1 load quantification. (**a**) Standard curves obtained using the standard primers and probe combination for the HIV-1 reference plasmid (▲ $y = -3.46 \log(x) + 39.26$; $R^2 = 0.999$), the plasmid from HIVBR025 clone 12 containing the 13G→A mutation (◇ $y = -3.48 \log(x) + 39.34$; $R^2 = 0.999$), the plasmid from HIVUG037 clone 1 containing the 14C→T mutation ($x\ y = -3.56 \log(x) + 40.12$; $R^2 = 0.997$) and the plasmid from HIV9611459 clone 2 containing the A insertion at position 9 (□ $y = -3.55 \log(x) + 41.19$; $R^2 = 0.992$). (**b** and **c**) Comparison between standard curves generated by using the standard mix (◇) and the modified mix (◆) for (**b**) plasmid from HIVUG037 clone 1 containing the 14C→T mutation (◇ $y = -3.56 \log(x) + 40.122$; $R^2 = 0.997$; ◆ $y = -3.54 \log(x) + 39.51$; $R^2 = 0.999$) or (**c**) plasmid from HIV9611459 clone 2 containing the A insertion at position 9 (◇ $y = -3.55 \log(x) + 41.198$ $R^2 = 0.992$; ◆ $y = -3.61 \log(x) + 39.553$ $R^2 = 0.999$). The standard curve obtained with the HIV-1 reference plasmid using the modified mix (Δ $y = -3.55 \log(x) + 38.79$; $R^2 = 0.999$) was included in both graphs **b** and **c** for comparison.

to determine the amount of DNA. On the instrument software, use the plate setup facility and define the appropriate wells as 'standards' and specify the concentration in those wells. The standard curve is then constructed automatically. It is realistic to expect a linear dynamic range of at least 6 logs with highly reproducible quantification; this defines the working dynamic range for the assay. One measure of the assay efficiency is obtained by comparison of the relative C_t values for subsequent dilutions of sample. The efficiency of the reaction can be calculated by the equation $E = 10^{(-1/\text{slope})} - 1$. The efficiency of the PCR should be as close as possible to 100%, corresponding to a doubling of the target amplicon at each cycle. Using this measure, an assay of 100% efficiency will result in a standard curve with a gradient of −3.323 (also see, http://www.gene-quantification.de/efficiency.html). The optimized assay will result in a standard curve with a slope between −3.2 and −3.5. Reproducibility of the replicate reactions also reflects assay stability, with R^2 values of 0.98 or above being indicative of a stable and reliable assay. The intercept on the C_t axis indicates the C_t at which a single unit of template concentration would be detected and is therefore an indication of the sensitivity of the assay. **Figure 4a** shows the standard curve of CCR5 assay: the equation

TABLE 6 | Comparison between real-time PCR results obtained with DNA extracted by the chloroform–phenol method (A) or by the crude lysate method (B) in a panel of clinical samples (1–6).

Sample	CCR5		HIV-1	
	C_t (±SD)	Cell number	C_t (±SD)	HIV-1 genomes per 10^5 cells
1				
Method A	22.20 (±0.16)	45,387	22.73 (±0.07)	139,363
Method B	22.25 (±0.11)	43,996	23.06 (±0.11)	117,114
2				
Method A	23.02 (±0.16)	25,833	26.94 (±0.13)	14,208
Method B	23.09 (±0.21)	24,613	27.02 (±0.40)	14,211
3				
Method A	23.92 (±0.18)	13,817	28.09 (±0.22)	12,235
Method B	24.23 (±0.06)	11,151	28.40 (±0.03)	12,345
4				
Method A	22.67 (±0.03)	32,793	33.20 (±0.32)	164
Method B	22.89 (±0.11)	28,263	33.24 (±0.57)	185
5				
Method A	24.04 (±0.06)	12,717	34.68 (±0.44)	156
Method B	24.05 (±0.01)	12,926	34.77 (±1.17)	148
6				
Method A	22.98 (±0.05)	26,558	37.61 (±1.96)	10
Method B	22.95 (±0.28)	27,115	36.85 (±1.08)	17

is $y = -3.48 \log_{(x)} + 38.197$, $R^2 = 0.999$; **Figure 4b** shows the quantification of HIV-1 reference plasmid with HIV Standard System in the presence of 1 µg of human DNA per reaction. As shown in **Figure 4b**, the HIV-1 system allows measuring a single viral genome for reaction, even in the presence of 1 µg of irrelevant human DNA (≈150,000 cells) without showing any problem of 'DNA mass effect'. This is a relevant feature of this assay, because the presence of human DNA mimics the real situation occurring in all biological samples.

Another important aspect of the above-described protocol is the possibility to detect most, if not all, group-M HIV-1 strains with very high accuracy, including those that seem to be present at low frequency (2%) in the infected population. To do this, we have optimized the HIV-1 Standard Assay calibrating the concentrations of forward primer (300 nM) and reverse primer (1,500 nM) and adding the new Reverse primer 9insA (300 nM) to the PCR master mix. This new system termed HIV-1 Modified Assay permits to detect with same efficiency isolates bearing mutations in the primer/probe sequences (i.e., HIVUG037, HIV9611459), without affecting the quantification of wild-type isolates (see **Fig. 5**). Finally, different methods for DNA preparation, including direct sample lysis, do not affect the accuracy of HIV-1 or CCR5 quantification (**Table 6**). Thus, the crude lysate method (option B) can be used as an alternative to the classical phenol–chloroform extraction, making it easier to export this technology to developing countries.

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