New Highly Sensitive Real-Time PCR Assay for HIV-2 Group A and Group B DNA Quantification

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ABSTRACT HIV-2 infection is characterized by a very low replication rate in most cases and low progression. This necessitates an approach to patient monitoring that differs from that for HIV-1 infection. Here, a new highly specific and sensitive method for HIV-2 DNA quantification was developed. The new test is based on quantitative real-time PCR targeting the long terminal repeat (LTR) and *gag* regions and using an internal control. Analytical performance was determined in three laboratories, and clinical performance was determined on blood samples from 63 patients infected with HIV-2 group A ($n = 35$) or group B ($n = 28$). The specificity was 100%. The 95% limit of detection was three copies/PCR and the limit of quantification was six copies/PCR. The within-run coefficients of variation were between 1.03% at 3.78 $\log_{10}$ copies/PCR and 27.02% at 0.78 $\log_{10}$ copies/PCR. The between-run coefficient of variation was 5.10%. Both manual and automated nucleic acid extraction methods were validated. HIV-2 DNA loads were detectable in blood cells from all 63 patients. When HIV-2 DNA was quantifiable, median loads were significantly higher in antiretroviral-treated than in naive patients and were similar for groups A and B. HIV-2 DNA load was correlated with HIV-2 RNA load ($r = 0.68$; 95% confidence interval [CI], 0.4 to 0.8; $P < 0.0001$). Our data show that this new assay is highly sensitive and quantifies the two main HIV-2 groups, making it useful for the diagnosis of HIV-2 infection and for pathogenesis studies on HIV-2 reservoirs.

KEYWORDS HIV-2, DNA, PCR, quantification

HIV-2 infection, mainly restricted to West Africa (1), is characterized by a slow disease progression associated with a slow decline in CD4 T cell counts (2, 3), a low rate of sexual or vertical transmission (4, 5), lower viral replication than HIV-1 (6, 7), and natural resistance to non-nucleoside reverse transcriptase inhibitors, fusion inhibitors (enfuvirtide), and several protease inhibitors, necessitating a specific approach for HIV-2-infected patient monitoring, different than that for HIV-1 infection (8, 9).

Most HIV-2-infected patients display undetectable plasma HIV RNA loads in the absence of antiretroviral therapy. In the French National HIV-2 Cohort, 71% of antiretroviral-naive patients had plasma viral loads below 100 copies/ml (10). Data from a West Africa cohort showed that 46.5% of antiretroviral-naive patients had undetectable HIV-2 RNA (<10 copies/ml), and 35.8% had low HIV-2 RNA loads of 10 to 100
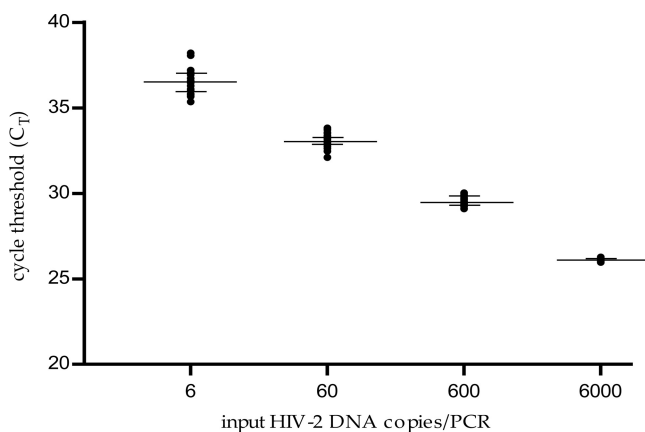
FIG 1 Standard curve of the HIV-2 DNA real-time PCR assay ($n = 22$ runs). The cycle threshold (C_T) is the number of cycles at which fluorescence passes a fixed limit (time to positivity). Median values and 25% and 75% interquartile ranges of the C_T are indicated (logarithmic scale).

copies/ml (11). In this context, HIV-2 DNA may be the only detectable viral marker for patients with undetectable HIV-2 RNA. It may then be a useful marker to confirm a diagnosis of monoinfection by HIV-2 or coinfection with HIV-1 in the case of strong serological cross-reactivity (12). This diagnosis will permit the adaptation of therapeutic decisions. Moreover, HIV-2 DNA detection is essential for the early diagnosis of infants born to HIV-2-seropositive mothers. Lastly, this marker can be useful for pathogenesis studies on HIV-2 reservoirs.

Several previous studies have developed HIV-2 DNA quantification assays, but they presented difficulties for quantifying group B viruses, and HIV-2 DNA was not detectable in some samples (13–15). No commercial HIV-2 DNA quantification assay is currently available.

The aim of the present study was to develop a reproducible, sensitive, and specific method for quantifying HIV-2 DNA, especially for the two endemic HIV-2 groups A and B (10), based on a real-time PCR method. The new test was developed and validated by three laboratories belonging to the Agence Nationale de Recherche sur le Sida et les Hépatites Virales (ANRS) AC11 Quantification Working Group to assess the analytical performance and interlaboratory reproducibility.

RESULTS

Analytical performance of the assay. All 50 HIV-1-positive and 30 HIV-negative DNA samples were negative in the assay, giving a specificity of 100%.

The standard curve showed strong linearity between the cycle threshold (C_T) values and $\log_{10}$ HIV-2 DNA copies/PCR, with a limit of quantification of six copies/PCR ($n = 22$ runs) (Fig. 1). The median correlation coefficient was 0.997 (range, 0.982 to 1), and the median slope was -3.45 (range, -3.11 to -3.64). The limit of quantification is 40 copies/10^6 cells (1.6 log) when studying 1 μg total DNA per PCR.

The analytical sensitivity of the assay was 100% at four copies/PCR (20/20), 95% at three copies/PCR (19/20), and 85% at two copies/PCR (17/20).

The within-run reproducibility was evaluated using the external standard with theoretical concentrations of 6,000, 600, 60, and 6 copies/PCR (3.78 $\log_{10}$, 2.78 $\log_{10}$, 1.78 $\log_{10}$, and 0.78 $\log_{10}$ copies/PCR, respectively). We obtained a mean of 3.80 $\log_{10}$ copies/PCR for the expected value of 3.78 $\log_{10}$ copies/PCR with a within-run coefficient of variation (CV) of 1.03% and mean values of 2.79 $\log_{10}$, 1.83 $\log_{10}$, and 0.85 $\log_{10}$ copies/PCR for the expected concentrations of 2.78 $\log_{10}$, 1.78 $\log_{10}$, and 0.78 $\log_{10}$ copies/PCR and within-run CVs of 1.60%, 3.43%, and 27.02%, respectively.

The positive control was determined to be 2.19 $\log_{10}$ copies/PCR for the between-run assays performed in the three laboratories, with a CV of 5.10%. This reproducibility was evaluated on DNA extracts.
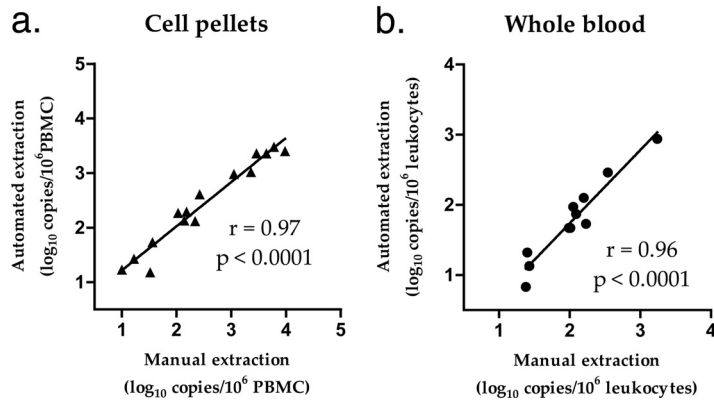
FIG 2 Comparison of manual and automated extractions from blood cell pellets and whole blood. (a) HIV-2 DNA was quantified in PBMCs (peripheral blood mononuclear cells [lymphocytes plus monocytes]) isolated from whole blood using Ficoll ($n = 15$). (b) HIV-2 DNA was quantified in leukocytes from whole blood, including PBMCs and polynuclear cells ($n = 11$).

The manual versus automated extractions were compared using samples extracted and quantified in parallel in labs A and C, respectively. The median values of HIV-2 DNA obtained from the 15 cell pellet samples were 2.34 $\log_{10}$ copies/10^6 peripheral blood mononuclear cells (PBMCs) with manual extraction and 2.29 $\log_{10}$ copies/10^6 PBMCs with automated extraction, with a median difference of 0.22 $\log_{10}$ and a correlation coefficient of 0.97 (95% confidence interval [CI], 0.92 to 0.99; $P < 0.0001$). The median values obtained from the 11 whole blood samples were 2.05 $\log_{10}$ copies/10^6 leukocytes with manual extraction and 1.73 $\log_{10}$ copies/10^6 leukocytes with automated extraction, with a median difference of 0.3 $\log_{10}$ and a correlation coefficient of 0.96 (95% CI, 0.85 to 0.99; $P < 0.0001$) (Fig. 2).

Clinical performance. The clinical performance was evaluated in lab C. All samples from HIV-2-infected patients were validated according to the internal control manufacturer's instructions.

Total DNA in the amounts of 122 to 1,000 ng (median, 548 ng) per PCR well was analyzed, depending on the total DNA concentrations in the extracts. HIV-2 DNA was detectable from all 63 patients. HIV-2 DNA was detectable but not quantifiable (<6 copies/PCR) from 20 patients (32%) and quantifiable ($\geq$6 copies/PCR) from 43 patients (68%), with a median HIV-2 DNA load of 2.45 $\log_{10}$ copies/10^6 PBMCs (interquartile range [IQR], 2.15 $\log_{10}$ to 3.00 $\log_{10}$). From the 20 patients with detectable but not quantifiable HIV-2 DNA, the same DNA extracts were retested using 2 to 6 PCR replicates. Eighteen samples gave positive results in all replicates, one sample had two positive results out of three, and one had three positive results out of four, at levels lower than six copies per PCR.

Among the 35 group A samples, HIV-2 DNA was quantifiable in 23 (66%), with a median load of 2.56 $\log_{10}$ copies/10^6 PBMCs (IQR, 2.29 $\log_{10}$ to 3.03 $\log_{10}$). Among the 28 group B samples, HIV-2 DNA was quantifiable in 20 (71%), with a median load of 2.27 $\log_{10}$ copies/10^6 PBMCs (IQR, 1.97 $\log_{10}$ to 2.81 $\log_{10}$). There was no difference between groups A and B in the proportions of patients displaying HIV-2 DNA loads below the limit of quantification ($P = 0.79$) nor in the median load (2.56 $\log_{10}$ versus 2.27 $\log_{10}$ copies/10^6 PBMCs, respectively; $P = 0.17$) when the load was quantifiable.

Among the 18 antiretroviral-naive patients, HIV-2 DNA was quantifiable from 10 (56%), with a median load of 2.08 $\log_{10}$ copies/10^6 PBMCs (IQR, 1.88 $\log_{10}$ to 2.28 $\log_{10}$). Among the 45 antiretroviral-treated patients, HIV-2 DNA was quantifiable from 33 (73%), with a median load of 2.60 $\log_{10}$ copies/10^6 PBMCs (IQR, 2.26 $\log_{10}$ to 3.09 $\log_{10}$). There was no difference in the proportions of patients displaying HIV-2 DNA loads below the limit of quantification between antiretroviral-naive and -treated patients ($P = 0.23$ for all patients, $P = 0.74$ for group A, and $P = 0.65$ for group B). The median HIV-2 DNA load was significantly higher in antiretroviral-treated than -naive patients ($P =$
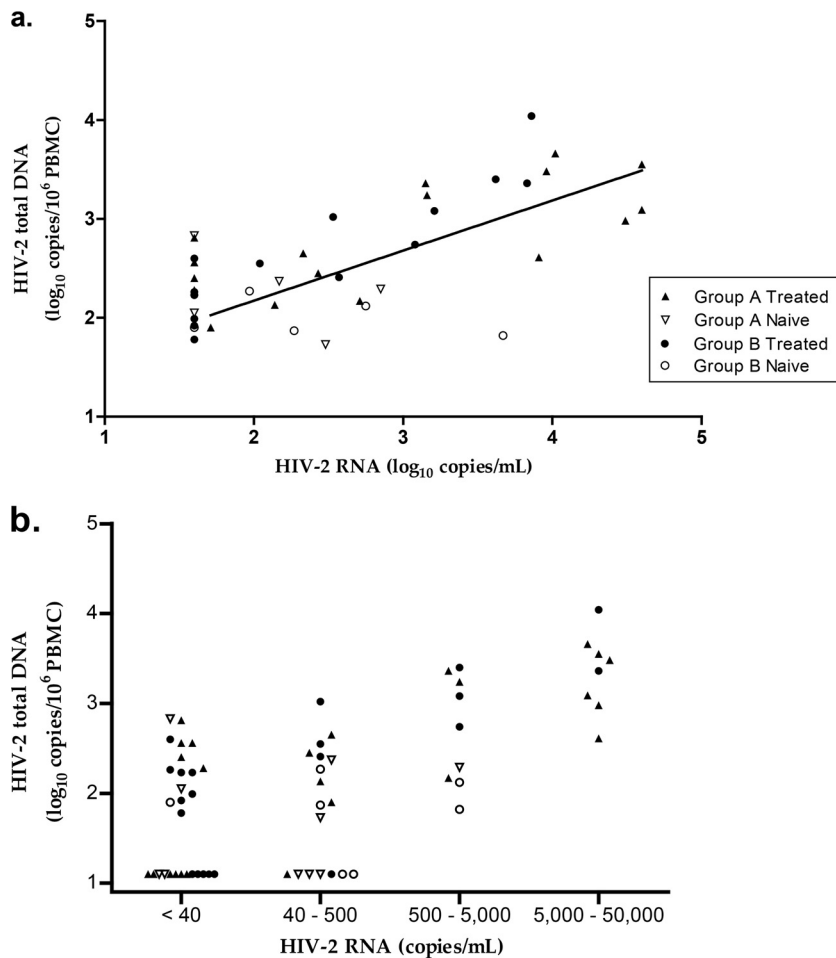
FIG 3 Clinical performance. (a) Correlation between HIV-2 DNA and HIV-2 RNA loads ($r = 0.68$; 95% confidence interval, 0.4 to 0.8; $P < 0.0001$ for the whole group). An arbitrary value of 1.60 $\log_{10}$ copies/ml was attributed to samples with an undetectable plasma viral load. (b) Distribution of HIV-2 DNA based on HIV-2 RNA concentration strata: <40, 40 to 500, 500 to 5,000, and 5,000 to 50,000 copies/ml.

0.003 for all patients, $P = 0.068$ for group A, and $P = 0.03$ for group B), when quantifiable.

When quantifiable, the HIV-2 DNA load correlated with the HIV-2 RNA load ($r = 0.68$; 95% confidence interval, 0.4 to 0.8; $P < 0.0001$ for the whole group and $r = 0.73$; 95% confidence interval, 0.4 to 0.9; $P < 0.0002$ for treated patients) (Fig. 3a). The proportions of patients displaying HIV-2 DNA loads below the limit of quantification were 46%, 39%, 0%, and 0% for HIV-2 RNA loads of <40 copies/ml, 40 to 500 copies/ml, 500 to 5,000 copies/ml, and 5,000 to 50,000 copies/ml, respectively. When quantifiable, the median (IQR) HIV-2 DNA loads were 2.26 $\log_{10}$ (2.02 $\log_{10}$ to 2.56 $\log_{10}$), 2.37 $\log_{10}$ (2.01 $\log_{10}$ to 2.50 $\log_{10}$), 2.74 $\log_{10}$ (2.17 $\log_{10}$ to 3.24 $\log_{10}$), and 3.42 $\log_{10}$ (3.06 $\log_{10}$ to 3.58 $\log_{10}$) copies/10^6 PBMCs, respectively (Fig. 3b). HIV-2 DNA loads were significantly different depending on the HIV-2 RNA load strata (analysis of variance [ANOVA], $P < 0.0001$). Comparisons between subgroups (e.g., group A and group B or antiretroviral-treated and -naive patients) according to HIV-2 RNA loads were not relevant because of the small numbers of patients in each subgroup.

DISCUSSION

Infection by HIV-2 is different from that by HIV-1, especially with respect to its slower progression, its therapeutic management, and its level of genetic diversity. Specific molecular methods are therefore necessary for the diagnosis and monitoring of HIV-2 infection (either alone or in the context of a coinfection with HIV-1), for the diagnosis

TABLE 1 Characteristics of the 63 HIV-2-infected patients[a]

Characteristic	Patients			P value[b]
	All ($n = 63$)	ARV naive ($n = 18$)	ARV treated ($n = 45$)	
Women (n [%])	39 (62)	14 (78)	25 (56)	0.15
Age (years) (median [IQR])	50 (43–55)	46 (42–52)	51 (43–58)	0.09
CD4 cell count (cells/mm³) (median [IQR])	485 (280–599)	603 (454–706)	424 (190–550)	**0.003**
HIV-2 RNA load detected				
Below the limit of quantification (n [%])	28 (44)	6 (33)	22 (49)	0.4
In patients with quantifiable viremia ($\log_{10}$ copies/ml) (median [IQR])	2.62 (2.16–3.64)	2.22 (1.96–2.65)	3.15 (2.48–3.88)	**0.01**
HIV-2 DNA load detected				
Below the limit of quantification (n [%])	20 (32)	8 (44)	12 (27)	0.23
In patients with quantifiable HIV-2 DNA ($\log_{10}$ copies/10^6 PBMCs) (median [IQR])	2.45 (2.15–3.00)	2.08 (1.88–2.28)	2.60 (2.26–3.09)	**0.003**

[a]ARV, antiretroviral; IQR, interquartile range; PBMC, peripheral blood mononuclear cell.
[b]Significant P values are in boldface font.

of infants born to seropositive HIV-2 mothers, and for studying the HIV-2 reservoir. Plasma viremia is undetectable in many HIV-2-infected patients in the absence of antiretroviral therapy, particularly in those with high CD4 T cell counts (11). HIV-2 DNA may be the only detectable marker in these patients.

The aim of this work was to develop a highly sensitive quantitative assay for HIV-2 DNA that is easy to implement in developing countries where HIV-2 is endemic.

This test exhibited 100% specificity. As expected, given the wide genomic divergence between HIV-1 and HIV-2, the HIV-2 primers did not hybridize to the HIV-1 genome. This assay also displayed good linearity (6 to 6,000 copies/PCR) and within-run reproducibility. We report good interlaboratory reproducibility. In addition, this assay that includes an external standard for quantification will improve the reliability of and the comparisons between studies. The excellent sensitivity (95% limit of detection, three copies/PCR) should prove useful both for clinical diagnosis and for pathophysiological studies. A minimal DNA input of 0.4 μg should be studied to have a 95% limit of detection of 50 copies/million cells.

Both manual and automated extraction methods, as well as two real-time PCR instruments, were validated for compatibility with local practices in resource-limited countries. We also showed that the assay can be performed on blood cell pellets and whole blood samples.

We used the same amplification protocol as used by the Generic HIV DNA cell kit (Biocentric, Bandol, France) to detect and quantify HIV-1 DNA (16, 17). This test is currently being used with success in many resource-limited countries (18). This will facilitate the use of this new assay to test for HIV-2 alone or jointly with HIV-1 in the same run. This will allow a reduction of analytical costs by increasing the number of samples per run and will facilitate the molecular diagnosis of dual HIV-1/HIV-2 infection within the same sample or the diagnosis of HIV infection in babies.

HIV-2 DNA was detectable in blood cells from all patients (infected either with HIV-2 group A or group B), owing to the use of primers and probes designed to efficiently amplify HIV-2 RNA (16); the sensitivity of this assay has thus been improved relative to previous assays which reported undetectable HIV-2 DNA in 17% (5/29) and 26% (11/42) of samples (13, 15). The addition of an internal control, which was absent in the previous assays (13, 14), allowed for the validation of the analytical process.

We also showed that HIV-2 patients receiving antiretroviral therapy had higher HIV-2 DNA loads than antiretroviral-naive patients, probably because patients who had been treated had more advanced disease, which necessitated the initiation of antiretroviral treatment. As described in Table 1, antiretroviral-treated patients had significantly fewer CD4 cells than antiretroviral-naive patients (424 versus 603/mm³, respectively; $P = 0.003$). Among the treated patients, higher HIV-2 DNA viral loads correlated

with higher HIV-2 RNA loads, similar to what has been reported for HIV-1 infection (18). A negative correlation between CD4 cell count and HIV-2 DNA load was also observed ($r = 0.66$; 95% confidence interval, 0.8 to 0.4; $P < 0.0001$).

The quantification of HIV-2 DNA levels would also provide information about the HIV-2 reservoir (19, 20), as HIV-1 DNA load has been reported to be a relevant marker of the HIV-1 reservoir (21–27).

In conclusion, this new HIV-2 DNA viral load assay has good analytical performance and clinical sensitivity. It is particularly successful for detecting both HIV-2 groups A and B, the most prevalent, more so than the previously described assays (13–15). It has been validated on a large well-characterized panel of patient samples. This easy-to-perform assay is appropriate for use in resource-limited countries in which multiple HIV-2 variants circulate. It will also be particularly useful for HIV-2 diagnosis in babies born to seropositive mothers and for the diagnosis of mono- or coinfections with HIV-1, which is important because of monitoring and the therapeutic consequences. It may also aid in pathogenesis studies on HIV-2 reservoirs, exploring new insights into the natural history of HIV-2 infection at different stages, and improving opportunities for clinical studies in treated patients.

MATERIALS AND METHODS

Patients samples. HIV-2 samples from 63 infected patients included in the French National HIV-2 Cohort (ANRS CO5) collected between June 2014 and May 2015 and with sufficient cells available were selected, and plasma HIV-2 RNA loads were determined as described previously (16). The viral genotype was representative of the genetic diversity among HIV-2 groups observed in the French cohort. Written informed consent was obtained from all patients at the time of inclusion in the cohort.

Whole blood sample aliquots collected with EDTA anticoagulant were frozen at −80°C. Blood cell pellets were obtained from whole blood after centrifugation (1,000 × g for 20 min) and plasma decantation. PBMCs were separated using a Ficoll-diatrizoate gradient (Eurobio, Courtaboeuf, France) from blood samples according to the manufacturer's instructions, and aliquots were frozen at −80°C.

Among the 63 patients assessed, 62% were women with a median age of 50 years (IQR, 43 to 55 years), and the median CD4 cell count was 485/mm³ (IQR, 280 to 599/mm³) (Table 1). Of these, 71% ($n = 45$) were treated by antiretroviral therapy, and the viral group, determined as previously described (28), was A for 35 patients (71% treated) and B for 28 (71% treated). The selected samples had the following HIV-2 RNA loads: <40 copies/ml ($n = 28$; 15 group A and 13 group B), 40 to 500 copies/ml ($n = 18$; 10 group A and 8 group B), 500 to 5,000 copies/ml ($n = 9$; 4 group A and 5 group B), and 5,000 to 50,000 copies/ml ($n = 8$; 6 group A and 2 group B). These samples were used to evaluate the clinical performance of the assay. Eleven whole blood samples from HIV-2-infected patients (6 group A and 5 group B) were also used to evaluate the performance using whole blood.

Blood samples from 30 HIV-negative subjects, 40 HIV-1 group M-positive patients, and 10 HIV-1 group O patients were tested to evaluate the specificity of the assay.

HIV-2 DNA quantification assay. Several extraction methods were used for whole blood and cell pellets, depending on the laboratory (three were involved in the study). For whole blood, total DNA was extracted from 200 μl using NucleoSpin blood kits (Macherey-Nagel, Düren, Germany) in labs A and B (Necker Hospital, Paris, and Charles Nicolle Hospital, Rouen, France) and using the QIAsymphony DSP DNA minikit (Qiagen, Courtaboeuf, France) in lab C (Bichat Claude Bernard Hospital, Paris). For cell pellets, the QIAamp DNA minikit and the QIAsymphony DSP DNA minikit were used for total DNA extraction from three to five million cells in labs A and C, respectively.

To normalize the HIV-2 DNA quantification, the amount of total DNA in extracts was determined by spectrophotometry (Nanodrop, Thermo Scientific, Wilmington, NC, USA) (labs A and B) or by quantification of the albumin gene (lab C) using the LightCycler FastStart DNA Master Hybprobe kit (Roche, Mannheim, Germany) and serial dilutions of human genomic DNA (Roche) as the standard (29, 30).

This new quantification assay is based on a triplex TaqMan PCR approach targeting the conserved consensus regions in the long terminal repeat (LTR) and *gag* gene already used in the HIV-2 RNA quantification assay (Biocentric) (16). It includes an internal control (yellow dye universal DNA extraction and inhibition control; Diagenode, Liège, Belgium) that is added before extraction. As recommended by Diagenode, the internal control is validated if positive, regardless of the cycle threshold.

The forward and reverse primers for the LTR region were 5′-AGCAGGTAGAGCCTGGGTGTT-3′ and 5′-TCTTTAAGCAAGCAAGCGTGG-3′, respectively (31), with an internal probe (5′-FAM-CTTGGCCGGYRCT GGGCAGA-BHQ1-3′ [FAM, carboxyfluorescein; BHQ1, black hole quencher 1]). The forward and reverse primers for the *gag* region were F3 (5′-GCGCGGAGAAACTCCGTCTTG-3′) and R1 (5′-TTCGCTGCCCACACA ATATGTT-3′), respectively, and the internal HIV-2 TaqMan *gag* probe was S65GAG2 (5′-FAM-TAGGTTAC GGCCCGGCGGAAAGA-BHQ1-3′) (32).

The reaction mixtures consisted of 50-μl volumes containing the DNA extracts (20 μl), primers and probes for HIV-2 (400 nM each), primers and probe for the internal control (1 μl), and 1× PCR buffer (2× qPCR MasterMix Plus; Eurogentec, Seraing, Belgium).

The thermocycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Amplification and data acquisition were carried out with the CFX96 (Bio-Rad,

Hercules, CA, USA) (Lab A) and TaqMan ABI 7900 and 7500 real-time PCR systems (Applied Biosystems, Courtaboeuf, France) (Labs B and C, respectively). The $\log_{10}$ numbers of targets initially present were proportional to the C_T values and were determined from the external standard curves.

DNA from HIV-2 (NIH-Z strain)-infected cells (Advanced Biotechnologies Inc., Eldersburg, MD, USA) was used as the external standard. This standard, evaluated at 131,300 copies/μl using a previously described assay (32), was first diluted in 200 ng/μl human genomic DNA (Promega, Madison, Wisconsin, USA) to a theoretical concentration of 60,000 copies/20 μl, followed by serial 10-fold dilutions in 25 ng/μl of human genomic DNA to concentrations from 6,000 copies/20 μl down to a final dilution of 2 copies/20 μl.

HIV-2 DNA levels were first reported as HIV-2 DNA copies/PCR. The numbers of copies of HIV-2 DNA/μg total DNA were then calculated using the extract concentrations, and the final results were reported as the number of copies/10^6 cells. The formula used to convert these results was HIV-2 DNA copies/μg total DNA $\times$ 1,000,000/150,000 $=$ HIV-2 DNA copies/10^6 cells (17, 33).

Analytical performance of the HIV-2 DNA quantification assay. The specificity was determined by testing blood samples from HIV-negative subjects (10 per lab), HIV-1 group M-positive patients (lab A), and HIV-1 group O patients (lab B).

The linearity was assessed in the three labs using serial dilutions of the external standard at 6,000, 600, 60, and 6 copies/20 μl (22 runs).

The analytical sensitivity was determined by testing dilutions of the external standards to 10, 6, 4, 3, and 2 copies/20 μl (20 replicates each) (lab A and lab B).

The within-run reproducibility was determined by testing the external standard at dilutions of 6000, 600, 60, and 6 copies/20 μl (10 replicates for each dilution) (lab B).

To determine between-run reproducibility, an HIV-2-positive control was prepared by diluting the DNA extract from cell cultures of an HIV-2 group A isolate (GenBank accession number M15390) to 2.13 $\log_{10}$ copies/20 μl by a previously described assay (32). This solution was tested in the three laboratories in separate runs ($n = 24$).

The manual and automated extractions were compared using blood cell pellets and whole blood; 15 PBMC pellets (8 group A and 7 group B) and 11 whole blood samples (6 group A and 5 group B) were extracted and quantified in parallel in labs A (manual) and C (automated).

Statistical analysis. Comparisons between groups were performed with the Mann-Whitney and Fisher exact tests. Pearson's correlation coefficients were calculated to estimate the relationship between the C_T values and $\log_{10}$ HIV-2 DNA copies/PCR, the relationship between the HIV-2 DNA and HIV-2 RNA loads, the relationship between the HIV-2 DNA and CD4 cell counts, and to compare manual versus automated extractions. An ANOVA was performed to evaluate the HIV-2 DNA values according to HIV-2 RNA strata.

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