Ultrasensitive Monitoring of HIV-1 Viral Load by a Low-Cost Real-Time Reverse Transcription-PCR Assay with Internal Control for the 5′ Long Terminal Repeat Domain

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Background: Current HIV-1 viral-load assays are too expensive for resource-limited settings. In some countries, monitoring of antiretroviral therapy is now more expensive than treatment itself. In addition, some commercial assays have shown shortcomings in quantifying rare genotypes.

Methods: We evaluated real-time reverse transcription-PCR with internal control targeting the conserved long terminal repeat (LTR) domain of HIV-1 on reference panels and patient samples from Brazil (n/H11549 1186), South Africa (n/H11549 130), India (n/H11549 44), and Germany (n/H11549 127).

Results: The detection limit was 31.9 IU of HIV-1 RNA/mL of plasma (>95% probability of detection, Probit analysis). The internal control showed inhibition in 3.7% of samples (95% confidence interval, 2.32%–5.9%; n/H11549 454; 40 different runs). Comparative qualitative testing yielded the following: Roche Amplicor vs LTR assay (n/H11549 431 samples), 51.7% vs 65% positives; Amplicor Ultrasensitive vs LTR (n/H11549 133), 81.2% vs 82.7%; BioMerieux NucliSens HIV-1 QT (n/H11549 433), 65.1% vs 65.4%; total (n/H11549 1450), 59.0% vs 63.8% positives. Intra-/interassay variability at medium and near-negative concentrations was 18%–51%. The quantification range was 50–10 000 000 IU/mL. Viral loads for subtypes A–D, F–J, AE, and AG yielded mean differences of 0.31 log10 compared with Amplicor in the 103–104 IU/mL range. HIV-1 N and O were not detected by Amplicor, but yielded up to 180 180.00 IU/mL in the LTR assay. Viral loads in stored samples from all countries, compared with Amplicor, NucliSens, or Versant, yielded regression line slopes (SD) of 0.9 (0.13) (P < 0.001 for all).

Conclusions: This method offers all features of commercial assays and covers all relevant genotypes. It could allow general monitoring of antiretroviral therapy in resource-limited settings.

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Antiretroviral therapy (ART) can effectively treat diseases caused by HIV-1 infection. Lower ART costs make treatment programs possible in nonaffluent countries, where 95% of HIV infections occur (1, 2). ART treatment should include accurate monitoring of plasma virus concentrations (viral load) (2–6). For prevention of mother-to-child transmission, ART treatment should be initiated if the viral load is above 1000 copies/mL in pregnancy, and continued for at least 4 weeks after delivery.
to-child transmission (PMTCT), virus detection is also required for testing newborns and mothers’ milk.

Testing for HIV-1 in the context of ART and PMTCT is best done by molecular methods, e.g., reverse transcription-PCR (RT-PCR), nucleic acid sequence–based amplification (NASBA), or branched DNA (bDNA) assays (4, 5, 7–10). Because of the high diversity of HIV-1, assay design is extraordinarily demanding. Therefore, viral-load testing relies almost exclusively on expensive commercial tests. With decreasing prices for drugs in many countries, therapy monitoring has become more expensive than the treatment itself (11, 12), a situation that leads to insufficient therapy monitoring, suboptimal patient management, and increased risk for emergence of drug-resistant virus strains. In addition, because they have been optimized for strains prevalent in the Northern Hemisphere, many commercial tests are not accurate for testing “exotic” HIV-1 subtypes found mainly in developing countries (13, 14). Moreover, some commercial assays are based on the gag gene, which is too variable for detection of outlier strains (15).

On the basis of our previous experience (15), we developed an inexpensive real-time RT-PCR viral-load assay that at least equals commercial tests with regard to technical features and performance. Instead of the gag gene, it targets the highly conserved long terminal repeat (LTR) region, thereby providing a spectrum of detectable and quantifiable genotypes beyond that of current assays.

**Materials and Methods**

**REFERENCE PLASMA**

We obtained WHO international standard reagent [National Institute of Biological Standards and Control (NIBSC)] (16) containing 100 000.00 IU of HIV-1, subtype B, per mL (16) and a subtype reference plasma panel (NIBSC) including lyophilized human plasma samples containing HIV-1 subtypes A, B, C, D, AE, F, G, H, N, and O. Another subtype reference plasma panel obtained from the National Reference Centre for Retroviruses included aliquots of human plasma containing HIV-1 subtypes A, B, C, D (2 samples), AE, F, G, and O (2 samples). Viral loads as determined by the Roche Amplicor system were provided with this panel.

**PATIENT PLASMAS AND COMMERCIAL VIRAL-LOAD ASSAYS**

Human plasma samples from Brazil (n = 1186), South Africa (n = 130), India (n = 44), and Germany (n = 127) were obtained from ongoing ART programs. All samples were anonymized, and ethics approval was obtained. South African samples were collected in Cape Town and tested by the NucliSens HIV-1 QT NASBA assay (BioMerieux, formerly Organon Technika) at the University of Cape Town. Indian samples were collected in Chennai and tested along with the samples from Frankfurt, Germany, by the Cobas Amplicor Monitor (Ver. 1.5) assay (Roche) at the University of Frankfurt. Brazilian samples were collected in Bahia (northeastern Brazil), Espiritu Santo (eastern Brazil), or Rio Grande do Sul (southern Brazil), respectively, and tested at these places by either the BioMerieux HIV-1 QT, Roche Amplicor, or Versant HIV-1 V 3.0 bDNA assay (Bayer). All materials were stored at −20 °C after initial viral-load testing and were transferred to the Bernhard Nocht Institute, where testing with the real-time LTR assay was done. LTR testing for all samples from Brazil was done by Brazilian laboratory staff during a 4-week training session at the Bernhard Nocht Institute.

The following quantification ranges were provided by the manufacturers of the commercial assays: Amplicor standard protocol, 400–750 000 IU/mL; Amplicor ultrasensitive protocol, 50–75 000 IU/mL; BioMerieux NucliSens, 80–10 000 000 IU/mL; Versant, 50–500 000 IU/mL.

**OLIGONUCLEOTIDE DESIGN**

A nucleic acid sequence alignment was set up that contained all LTR sequences present in the Los Alamos National Laboratory database by 2002 (reproduced in Electronic File 1, which can be found in the Data Supplement accompanying the online version of this article at http://www.clinchem.org/content/vol52/issue7/). Three conserved domains, necessary for binding of a probe and 2 flanking primers, were identified on inspection of the alignment, as current primer design software (Primer Express; Applied Biosystems) yielded no results. Two different candidate probes were defined in the middle conserved domain (region 575–612). In the flanking potential primer-binding sites (regions 520–548 and 625–653), up to 10 different variations of oligonucleotides per site were ordered and tested experimentally in each possible combination with both of the 2 candidate probes. The most efficient combination of oligonucleotides showed 3 mismatched nucleotide (nt) residues in the probe-binding domain in HIV-1 subtype O (nucleotides 580, 586, and 587), which were compensated for by use of an additional 5′-nuclease probe (see below). Because of the high degree of conservedness at the chosen oligonucleotide binding domains, no additional mismatch compensation was necessary.

**LYOPHILIZED FULL-VIRUS QUANTIFICATION CALIBRATOR**

A storable, calibrated quantification calibrator was generated for routine use. Cell-culture–derived HIV-1, subtype B, strain NL4–3, was inoculated in fresh-frozen human plasma, diluted, and inoculated in volumes of 10 μL in 200 μL of buffer AVL. RNA extraction and real-time RT-PCR testing were conducted as described below. After an initial limiting dilution series, concentrations of 1, 2, 3, and 4 log10 above the detection limit were defined as 1, 10, 100, and 1000 PCR units (PCRU), respectively. Stocks of these concentrations were generated in large volumes, divided into 10-μL aliquots, and lyophilized in vacuum.
glass tubes. To calibrate the stocks, the contents of each of 4 replicate glass tubes of each concentration were completely redissolved in 200 μL of buffer AVL and extracted as described above. In parallel, 4 replicate samples of human plasma containing 5000 IU of WHO HIV-1 international standard were treated like patient plasmas and tested by the ultrasensitive protocol. After obvious outliers were eliminated, we defined the lyophilized plasma samples as calibrators in the real-time PCR software and entered concentrations in terms of PCRU. The mean PCRU value obtained for the WHO standard was then used to determine a correction factor for the conversion of PCRU to WHO standard IU values. The correction factor was readjusted for each new lot of stock preparations. For up to at least 4 months, the maximum storage duration, no loss in RNA concentration was perceivable. Systematic storage studies were not done because the stability of HIV-1 and other enveloped viruses in lyophilized plasma has been confirmed several times [see, for example, Refs. (16–20)].

SYNTHETIC HIV-1 RNA
The LTR PCR fragment (see below) was ligated into a pCR 2.1 plasmid vector and cloned in Escherichia coli by means of a pCR 2.1-TOPO TA cloning reagent set (Invitrogen). Plasmids were purified, sequenced, and reamplified with plasmid-specific primers (M13f-20 and M13r, from the reagent set) to lower the plasmid background in subsequent in vitro transcription. Reamplification products were transcribed into RNA with the MegaScript T7 in vitro transcription kit (Ambion) (15, 21, 22). After DNase I digestion, RNA transcripts were purified with Qiagen RNeasy columns and quantified spectrophotometrically. Results from RT-PCR with primer LTRAs4 and other enveloped viruses in lyophilized plasma has been confirmed several times [see, for example, Refs. (16–20)].

INTERNAL CONTROL
An alternative probe-binding site was introduced at HIV-1 nt positions 580–605 (numbering according to HIV-1 strain HXB2), removing the binding site of the HIV-1–specific probe at the same time. According to a previously described strategy (22), 2 partial amplicons were generated by use of primer IcS (5′-CAGGAGTGTATTGGGAAATCAAGATGCATCTTATTTTATGGAAAAGATTTAA-3′) in combination with primer LTRAs4 (sequence below), and IcAs (5′-GCATCTGATTCCCATCATTCTTGGATACTACCTCACTCCAGTTT-CGAGCCT-3′) in combination with primer LTR3S (sequence below), respectively. After extension-PCR with primers LTR3S/As4, products were TA-cloned, purified, sequenced, and transcribed as described previously (15, 21, 22). The RNA/DNA ratio, as determined by PCR and RT-PCR, was 106. The resulting synthetic RNA was termed srLTRic. With up to 160 copies of srLTRic added, a calculated 4.8 copies of HIV-1 RNA were still detectable in the same reaction in 5 of 5 parallel assays. From 320 copies of internal control onward, signal strength for HIV-1 decreased. Eighty copies of srLTRic per reaction was chosen as the working concentration, which did not interfere with the detection of 50 IU/mL HIV-1 in 24 of 24 reactions, as confirmed by testing of the WHO international standard.

ULTRASENSITIVE EXTRACTION OF HIV-1 RNA FROM PLASMA
We treated 1.2 mL of cleared plasma with centrifugation for 1 h at 21 000g in a benchtop centrifuge. Supernatants were removed completely, and virus pellets were lysed directly in 200 μL of buffer AVL (Qiagen Viral RNA Mini Kit). To the lysed pellets we added 200 μL of ethanol (990 mL/L); after mixing, we loaded the mixture on a Qiagen viral RNA minicolumn. Elution was with 50 μL of buffer AVE at 80 °C. In routine testing, buffer AVL was supplemented with internal control srLTRic at 2000 copies/mL (see below) and stored for up to 5 days at room temperature.

QUANTIFICATION OF HIV-1 VIRAL LOAD BY ONE-STEP REAL-TIME RT-PCR
A 50-μL reaction contained 10 μL of RNA extract, 1× reaction buffer (Access RT-PCR reagent set; Promega), 2 mM magnesium sulfate, 200 μM each deoxynucleotide triphosphate, 600 nM primer LTR S4 (5′-AACCTCAATAAAGCATTGGCGCTGA-3′; nt 520–543 of HIV-1 B reference strain HXB2), 400 nM primer LTR As3 (5′-GTTCGCGGCCACTCTGACATG-3′; nt 629–647), 50 nM probe LTRP1 (5′-FAM-TCTGGTACTAGATCCCCTCAGACC-Black Hole Quencher 1–3′, where FAM is 6-carboxyfluorescein; nt 580–605), 50 nM probe LTRP2 (5′-FAM-CCTGGTGACTAGATCCCCTCAGACC-Black Hole Quencher 1-3′; nt 580–605), 100 nM probe YFPY (5′-Yakima Yellow-ATCGGTTCGTTGAGCGATTAGCAG-Black Hole Quencher 1-3′; nt 580–605), 100 nM probe AVE (5′-FAM-CTGTGGTGACTAGATCCCCTCAGACC-Black Hole Quencher 1-3′; nt 580–605), 100 nM each deoxynucleotide (FAM, VIC®, and ROX internal reference dye (all dyes, primers, and probes from Tib Molbiol)).

Thermal cycling was as follows: 48 °C for 20 min; 94 °C for 2 min; 12 cycles of 95 °C for 10 s and 62 °C for 30 s; 40 cycles of 94 °C for 10 s and 56 °C for 40 s. Cycling was performed in either Applied Biosystems 7700 or 7000 SDS instruments with no perceivable difference in results. Fluorescence was read out in the 56 °C step of the final segment of the cycling program. The fluorescence at the VIC® and FAM wavelengths was analyzed separately with (ABI 7700) or without (ABI 7000) normalization with the ROX dye. In the ABI 7700, the baseline area was routinely defined in cycles 1 to 11. In the ABI 7000, the “auto baseline” mode was used for data analysis. In general, calibration curves were used as automatically
obtained from the operation software of the real-time RT-PCR systems. In some individual runs, manual readjustments of the threshold line had to be made according to the fluorescence noise in the negative controls. When the low–copy-number reaction of the calibration curve failed, this was taken as a sign of insufficient sensitivity, and the whole run was repeated. All patient data presented are for single determinations, i.e., no duplicate testing was done.

For those reactions that did not yield FAM amplification signals, the VIC amplification signal was inspected. When the signal was negative or was delayed against the mean VIC signal in the negative plasma controls by 2 or more cycles, the respective sample was considered invalid because of PCR inhibition. Such samples were reprocessed and retested in original concentration as well as after dilution 1:10 in plasma from an HIV-negative donor.

STATISTICAL METHODS
The Statgraphics plus (Ver. 5.1) software package (Manugistics) was used for all statistical analyses described.

Results
Because of HIV-1 variability, primer design for the real-time LTR RT-PCR was based on a comprehensive nucleic acid alignment with binding domains of maximal conservedness. We optimized reaction chemistry and implemented a thermal cycling protocol with a precycle element to increase the amplification stringency (23). After optimization, tests showed high sensitivity with synthetic HIV-1 RNA srLTRw, with detection of 1–5 copies per reaction.

We tested the quantification range with a dilution series of srLTRw. Up to $4 \times 10^9$ copies of RNA per reaction, corresponding to 1.6 $\times 10^7$ copies/mL of plasma sample, were quantified with a correlation coefficient between expected logarithmic copy numbers and observed threshold cycle values of 1.0.

To prevent PCR inhibition from interfering with accurate quantification, we designed a competitive internal control that used the same amplification primers as HIV-1 and was detected by a probe of different sequence composition and different fluorescent labeling. The internal control was added to all reactions at the same step as the addition of lysis buffer. After extensive experimental evaluation, we adjusted the working concentration to 80 copies per assay, or 400 copies per RNA preparation.

We determined the assay limit of detection by diluting the WHO HIV-1 international standard in human frozen plasma to 11, 33, 100, 1000, 10 000, and 100 000 IU/mL. Plasma containing each concentration was extracted in 4 parallel ultrasensitive RNA preparations, and each of these was tested in 4 replicate RT-PCR assays, 16 data points per concentration. No dropout in HIV-1 detection in 16 parallel reactions was observed from 100 copies/mL upward. We performed Probit regression analysis to determine the projected response rates according to a dose–response model (Fig. 1). At a concentration of 31.9 IU/mL, detection probability was 95% or higher (95% confidence interval, 24.8–48.1 IU/mL). The limit of detection was thus slightly higher than that of several commercial assays for HIV-1 viral load.

We assessed intraassay accuracy with 8 replicate results for an HIV-1 B plasma containing 1200 IU/mL tested in the same experiment. The mean viral load was $1117.5 \pm 10^9$ IU/mL, with a CV of 18% and an SD of 0.09 $\log_{10}$. To confirm that the assay accurately quantified viral loads close to the detection limit, we diluted the WHO international working reagent to 50 IU/mL and assayed it in 18 replicate tests in the same experiment. The mean measured value was 51.4 IU/mL with a CV of 51% and an SD of 0.27 $\log_{10}$. Because this deviation was compatible with clinical application, the lower limit of the quantification range was defined at 50 copies/assay. Considering the upper limit of the 7700 SDS operation software in analyzing high concentrations without a need to change analysis settings, the total quantification range was defined as 50–10 000 000 IU/mL.

We assessed interassay accuracy by testing 2 plasma samples to which HIV-1 B had been added at 9600 and 960 IU/mL, respectively, in 20 different experiments each. The mean measured quantities were 10 656 and 986 IU/mL, respectively. The CVs were of 39% and 41%, respectively, with SDs of 0.17 and 0.23 $\log_{10}$.

Because our assay was intended to detect a broader spectrum of viral subtypes than gag-based assays, we assessed the efficiency with which different subtypes of HIV-1 were amplified by testing 2 different subtype reference panels containing all relevant strains of HIV-1 groups M, O, and N and 12 plasma samples from patients infected with non-B subtypes, which provided genotypes not available in the reference panels, e.g., circulating recombinant forms AE and AG and subtype J.

Fig. 1. Probit regression analysis to determine the limit of detection of the LTR assay.
Probability (y axis) is plotted against RNA concentration in 16 parallel test samples per data point (x axis). The plot depicts the observed proportion of positive results in parallel experiments ( ), as well as the derived predicted proportion of positive results at a given input concentration of RNA. The solid line is the prediction, and the dashed lines are the 95% confidence limits for the prediction. Note that the tested range of concentrations extends farther than shown here (see the text).
We expected test variation to occur preferably in the low and medium ranges of virus concentrations; we therefore diluted high-concentration reference samples in human fresh-frozen plasma to concentrations of 1000–10 000 copies/mL and then measured viral loads with the Roche Cobas Amplicor (Ver. 1.5) and LTR assays (Fig. 2).

Fig. 2. Testing of 2 different subtype reference panels. (A), Panel 1, German National Reference Centre for Retroviruses; Panel 2, NIBSC panel. (B), plasma samples from patients with non-B HIV-1. ■, viral load as determined by real-time RT-PCR LTR assay; □, viral load as determined by Roche Cobas Amplicor (Ver. 1.5). The log_{10} differences in viral loads are plotted at the bottom of both panels. Letters displayed along the x axis of each panel are identifiers of HIV strains present in the tested patients. *, not detected.
None of the samples containing HIV-1 subtypes O and N was detected by the Roche Amplicor, but all yielded viral-load results with the LTR assay. When we omitted the subtype N and O samples, the mean logarithmic viral loads were almost identical with both assays (3.57 and 3.60 by the Amplicor and LTR assays, respectively; difference insignificant in the t-test and Wilcoxon two-sample test). In most individual samples, the differences of viral loads were $<0.5 \log_{10}$ (Fig. 2). Viral loads in individual samples contained in the reference panels differed by 0.26
log_{10}, on average; those in individual patient samples differed by 0.37 log_{10}, on average. In all samples, the mean difference of paired viral loads was 0.31 log_{10}.

We next determined the quantitative correlation with other viral-load assays. Because our assay was intended for application in resource-limited countries with high HIV-1 prevalence, evaluation included patients from such regions. Stored samples from Germany (n = 127), Brazil (n = 1186), South Africa (n = 130), and India (n = 44) were available. All samples had been tested previously by either the Cobas AmpliCord Monitor (Ver. 1.5) RT-PCR assay from Roche, the NucliSens HIV-1 QT NASBA assay from BioMerieux, or the Versant HIV-1 bDNA assay from Bayer. All samples were then quantified by real-time LTR RT-PCR. For regression analyses, those samples that had results below or above the quantification ranges of either the comparison method or the LTR assay were excluded. As shown in Fig. 3, LTR real-time RT-PCR correlated well with all of the commercial viral-load assays. Regression line slopes in all sample panels were in the range (SD) of 0.9 (0.13), indicating highly significant correlations (P < 0.001 for all).

Because regression analysis can only compare samples in which results from both tests are within the quantification ranges of each test, respectively, it did not reflect differences in overall clinical sensitivity. To compare the detection rates of the LTR assay with those of commercial tests, we collected qualitative results from all available samples, as shown in Table 1. The rate of positive results in all (n = 43) samples tested with the Roche AmpliCord conventional protocol was 51.7% compared with 65% when the same samples were tested with the real-time LTR assay. The corresponding numbers (percentage positive with the comparison method/percentage positive with LTR assay) for the other methods were as follows: Roche AmpliCord ultrasensitive (n = 133 samples), 81.2% vs 82.7% for the LTR assay; NucliSens QT (n = 453), 60.5% vs 65.1%; Bayer Versant (n = 43), 57.7% vs 55.4%; total (n = 1450), 59.0% vs 63.8%. Thus, only the Bayer Versant appeared to be slightly more sensitive than the LTR real-time RT-PCR when used for testing clinical samples.

We analyzed 40 different routine quantification runs to determine the rate of inhibited reactions that occur during routine viral-load monitoring. Of the 454 samples that did not yield an HIV-1 signal in these runs, 17 had an undetectable internal control, or 3.7% inhibited reactions (95% confidence interval, 2.32%–5.9%) in which a false-negative result would have been obtained had the internal control not been included.

To demonstrate the applicability of the new assay for routine monitoring of patients on ART, we selected 5 individuals in whom viral loads were determined by LTR real-time RT-PCR before and after start of therapy (Fig. 4). Over the whole range of viral loads, no predilution of samples was necessary; all samples were well within the dynamic range of the assay.

**Discussion**

ART is now becoming an affordable option in resource-limited countries, where most HIV-1 infections occur, but without viral-load monitoring, the spread of drug-resistant virus strains has to be anticipated (12, 24). Unfortunately, current commercial viral-load monitoring assays were designed for use in affluent industrialized countries and are thus optimized to work best on HIV-1 subtype B (13, 14, 25, 26).

Our study results show that LTR is a highly suitable target gene for quantification of exotic HIV-1 genotypes, thereby reducing the risk of genotype bias. We tested all important genotypes of HIV-1, and except for groups N and O, the overall results of virus quantification with different assays were highly concordant (27–30).

Inclusion of an inhibition control is important because inhibited tests cause false results in virus screening. Our rate of 3.7% inhibited tests is in good concordance with the few previous studies that have addressed this issue systematically (15, 31–34). The accuracy of our assay was equivalent to or better than values observed in earlier studies on commercial and in-house viral-load assays (28, 35–37), and the good analytical sensitivity of our assay allowed a broad quantification range covering the range of viral loads in both treated and untreated patients.

The major benefit of our method is cost reduction. Commercial assays are currently offered, even in developing countries, at prices of approximately US $50.00–$100.00 per test; for our assay the net price is approximately US $10.00. With full PCR license fees, costs would still be approximately US $20.00 per test. Our test is not a Communauté Européenne-certified in vitro diagnostic method, however, and thus cannot be used for commercial diagnostic services under European Union law.

Screening of newborns is required in the context of PMTCT in developing and emerging countries; therefore,

### Table 1. Detection of HIV-1 by real-time LTR RT-PCR assay and by commercial methods.

| Result constellation | Comparison method used |
|----------------------|------------------------|
|                     | Real-time LTR assay    | Amplicor standard | Amplicor ultrasensitive | NucliSens QT NASBA | Versant | Total |
| Positive Positive   | Positive               | 222               | 108                      | 257                | 232     | 819   |
| Negative Negative   | Negative               | 149               | 23                       | 141                | 175     | 488   |
| Positive Negative   | Negative               | 1                  | 0                        | 17                 | 18      | 36    |
| Negative Positive   | Positive               | 59                 | 2                        | 38                 | 8       | 107   |
| Total               |                        | 431               | 133                      | 453                | 433     | 1450  |
many patients require only qualitative PCR testing. Because most commercial PCR assays do not allow qualitative testing without unnecessary extra information, which would be less expensive, high costs are a potential barrier to PMTCT. Our assay can be used for screening of more than 90 samples per run in a purely qualitative mode because of its open format. In this mode, the same reliability and sensitivity are provided as in viral-load monitoring, with the internal control to assure maximum sensitivity in each individual reaction.

In conclusion, our method is a highly cost-efficient viral-load monitoring assay with an open format and reliable day-to-day operation. In our hands the assay has been running reliably for 3 years of routine testing. The evaluation cohorts in our study may require alternative viral-load assays in the near future. South Africa has the largest population of HIV-infected patients in the world (38, 39) and has a nationwide public service ART program underway (40, 41), requiring urgent reduction in the cost of viral-load monitoring. The situation is similar in Brazil, where the absolute number of patients is high and the government is making highly active ART universally available (42, 43). Finally, India has one of the fastest growing populations of HIV-1-positive individuals, and emerging ART programs will require affordable viral-load testing (44, 45). Considering the large and increasing populations of individuals infected with HIV-1 in resource-poor settings, the potential benefit of an affordable viral-load assay is enormous. The need for alternative methods is obvious, and our approach might contribute to making this aim attainable.

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