Development and Evaluation of an Affordable Real-Time Qualitative Assay for Determining HIV-1 Virological Failure in Plasma and Dried Blood Spots

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Virological failure (VF) has been identified as the earliest, most predictive determinant of HIV-1 antiretroviral treatment (ART) failure. Due to the high cost and complexity of virological monitoring, VF assays are rarely performed in resource-limited settings (RLS). Rather, ART failure is determined by clinical monitoring and to a large extent immunological monitoring. This paper describes the development and evaluation of a low-cost, dried blood spot (DBS)-compatible qualitative assay to determine VF, in accordance with current WHO guideline recommendations for therapy switching in RLS. The assay described here is an internally controlled qualitative real-time PCR targeting the conserved long terminal repeat domain of HIV-1. This assay was applied to HIV-1 subtypes A to H and further evaluated on HIV-1 clinical plasma samples from South Africa (n = 191) and Tanzania (n = 42). Field evaluation was performed in Uganda using local clinical plasma samples (n = 176). Furthermore, assay performance was evaluated for DBS. This assay is able to identify VF for all major HIV-1 group M subtypes with equal specificity and has a lower detection limit of 1.00E+03 copies/ml for plasma samples and 5.00E+03 copies/ml for DBS. Comparative testing yielded accurate VF determination for therapy switching in 89% to 96% of samples compared to gold standards. The assay is robust and flexible, allowing for “open platform” applications and producing results comparable to those of commercial assays. Assay design enables application in laboratories that can accommodate real-time PCR equipment, allowing decentralization of testing to some extent. Compatibility with DBS extends access of sampling and thus access to this test to remote settings.

In 2010, the HIV-1 epidemic was estimated to include 34.0 million (range, 31.6 to 35.2 million) infected adults and children across the globe. An alarming 67.4% (n = 22.9 million) of the total global infections are people residing in sub-Saharan Africa. As a result of antiretroviral therapy (ART) scale-up initiatives, 6.65 million infected individuals requiring treatment were receiving it in sub-Saharan Africa by the end of 2010 (1). However, particularly in resource-limited settings (RLS), effective treatment faces challenges, which include failing supply chains resulting in drug shortages, drug toxicity of older first- and second-line drugs, failure of patient adherence, drug interactions, lack of qualified health care staff, or failing adherence support, etc. As a result, HIV-1 can develop drug resistance to ART, leading to virological failure (VF) and subsequently ART failure. A recent report has shown that the prevalence of pre-ART HIV-1 drug resistance in 13 sites in various countries in sub-Saharan Africa is 5.6%, ranging from 1.1% in South Africa to 12.3% in Uganda (2). Recent scientific findings have led to the consideration of “treatment as prevention,” which according to the most intensive “test and treat” scenario could ultimately increase the number of HIV patients qualifying for ART to 32 million (3). With rapidly increasing numbers of HIV patients on ART in RLS with weak health systems, the risk of further increase of HIV-1 drug resistance is imminent.

The success of increased access to ART in RLS has largely been due to massive donor funding and important reduction of costs of selected first-line drugs. However, reduced susceptibility to these first-line drugs and the consequent switching to second-line drugs would at least partly undo early ART successes and result in higher expenditures and increasing numbers of patients on failing regimens with no options for effective second-line or salvage therapies (4, 5).

According to the definition of the WHO, VF is a repeated viral load of ≥5.00E+03 RNA copies/ml in an individual taking ART for at least 4 to 6 months (6). Timely detection of VF by viral load (VL) testing, which is routine in industrialized countries (7), is necessary to prevent accumulation of HIV drug resistance (8) or to identify poor adherence to the treatment. However, in RLS, high costs and technical complexity limit VL monitoring, and treatment failure is determined primarily by clinical monitoring for stage three and four AIDS-defining illnesses and, if available, immunological monitoring using CD4 counts (6). The inadequacies of CD4 counts for determining true treatment failure have been described on many occasions (9–12). The clinical-immunological monitoring approach results in individuals being left on suboptimal regimens for an extended period of time with the risk...
of accumulating drug resistance mutations or unnecessary switching to second-line therapy based on non-VL-supported decisions (4). Both scenarios limit future treatment options and may cause increased costs associated with second-line therapy (5).

The current paper addresses the challenge of determination of VF in RLS by taking several premises into consideration that reflect the actual public health situation in these settings. First of all, the standpoint was taken that determination of an exact VL is not required to determine ART failure, and therefore a less complex, and thus less expensive, assay that classifies a sample as either above or below a treatment success threshold would suffice. Second, in order to implement the WHO recommendations of task shifting and decentralization of ART to remote settings, the consequence would be that complex procedures, including drawing blood, isolation and storage of plasma samples, and cold chain shipments to qualified labs for VL testing, should be avoided. Rather, VF should be detectable on dried blood spots (DBS), a sampling alternative that is inexpensive and easy to collect and transport and has proven application for VL testing (13, 14). Third, given the fact that for accurate detection of VF, a nucleic acid amplification step remains necessary and taking into consideration the realities of contamination risks in remote labs, it was decided to concentrate on a real-time PCR approach. This allows for VF determination in a closed system and with equipment that is continuously evolving, regularly reducing in price, and being adapted to local circumstances through battery and solar energy applications. Finally, it was considered essential that the protocol for VF testing be specific, “open platform,” applicable on a wide array of real-time PCR instruments in various African settings, and freely available in the public domain.

With the above assumptions in mind, the Affordable Resistance Test for Africa (ARTA) consortium was established, consisting of a unique combination of academia, industry, and nongovernment organizations both in Africa and Europe (the members of the consortium are listed in the Acknowledgments). Here we report on the results of ARTA research to develop a real-time PCR assay that can be used as a screening tool to determine VF in RLS. This virological failure screening assay (VFA) can be readily applied in basic laboratories, using either plasma samples or DBS as the sample input. The VFA is applicable for all major HIV-1 group M subtypes and is specifically designed to identify VF as defined by the WHO as a VL of ≥5.00E+03 copies/ml (6).

**MATeRIALS AND Methods**

**Samples.** (i) HIV-1 subtype reference panel. A panel of virus isolates consisting of HIV-1 subtypes A through H (Table 1) was obtained from BBI Biotech Research Laboratories Inc. (Gaithersburg, MD, USA) for assay optimization and evaluation at the University Medical Centre Utrecht (UMCU), Utrecht, the Netherlands. Serial dilutions were prepared from these stocks using HIV-1-negative human plasma samples. These dilutions were also used to spike HIV-1-negative whole-blood samples for dried blood spot (DBS) preparation.

(ii) Clinical samples. Clinical plasma samples from HIV-1-infected individuals from several African sites were included for further evaluation at the UMCU, Utrecht, the Netherlands. Samples were selected to include several subtypes with a variety of viral loads (VL) in accepted ranges for subsequent analysis. Samples from South Africa (n = 191) were plasma samples sent for routine VL testing, performed on the Cobas Ampliprep/Cobas TaqMan system v2 (Roche, Penzberg, Germany), and represented HIV-1 subtype C with a VL range of 1.30E+03 to 3.00E+06 (median, 5.50E+04) copies/ml. Samples from Tanzania (n = 42) were part of an ongoing prevention-of-mother-to-child-transmission (PMTCT) study (15), where VL was determined using the COBAS Amplipcr HIV-1 Monitor test v1.5 (Roche). Samples included subtypes A (n = 23), C (n = 10), and D (n = 6), and three samples with undetermined subtype with a VL range of 6.6E+02 to 3.0E+05 (median, 2.67E+04) copies/ml.

In addition, as part of a technology transfer program, the assay described here was applied in three Joint Clinical Research Centre (JCR) laboratory sites in Uganda, where retrospective plasma samples collected from HIV-1-positive individuals as part of the PharmAccess African Studies to Evaluate Resistance (PASER) program were included (16). These samples represented baseline and follow-up clinical samples at yearly intervals after therapy initiation. For these samples, routine VL testing had been performed in Uganda using the Cobas Ampliprep/Cobas TaqMan system v2 (Roche). A total of 176 plasma samples were tested, including subtypes A (n = 89) and D (n = 64) and 23 with an unknown subtype, with a VL range of 1.00E+02 to 1.00E+06 (median, 1.00E+04) copies/ml. Twenty-five confirmed HIV-1-negative plasma samples were included for assay specificity control.

To investigate the application of the assay with DBS samples, DBS were prepared from EDTA-collected whole-blood samples for participants of the PASER program (16). The same blood sample was then centrifuged, and the plasma was removed for analysis. These samples are subsequently referred to as paired plasma and DBS samples. A total of 82 paired samples were tested in Uganda, with a VL range of 4.40E+01 to 7.18E+06 (median, 2.61E+03) copies/ml. DBS samples were stored at −70°C for 270 to 515 (median, 485) days (n = 31), −20°C for 45 to 112 (median, 82) days (n = 21), or room temperature for 2 to 192 (median, 126) days (n = 30).

(iii) Internal control. An internal control (IC) was added to each clinical sample at a fixed amount of 10% of the elution volume at the start of nucleic acid isolation. The IC consisted of the nonhuman RNA virus encephalomyocarditis virus (EMCV) and was prepared at the UMCU, Utrecht, the Netherlands, in batches of single-use aliquots and stored at −80°C until use.

**Virological failure screening assay (VFA).** (i) Nucleic acid isolation from plasma samples. At the UMCU, Utrecht, the Netherlands, viral nucleic acid (NA) isolation was performed using NucliSens magnetic extraction reagents in combination with the MiniMag (bioMérieux, Boxtel, the Netherlands). For each sample, an input of 100 μl plasma was used, or two DBS of 50 μl each, and 2.5 μl IC. Positive and negative controls were included in each run. Upon completion of the isolation procedure, purified nucleic acids were eluted in 25 μl elution buffer.

In Uganda, NA isolation was performed using the QIAamp RNA kit (Qiagen GmbH, Germany) per the manufacturer’s instructions. Input was 100 μl of plasma, or two 50-μl DBS, and 5 μl IC. Isolated NA was eluted in 50 μl elution buffer.

Upon completion of both isolation procedures, the eluates were used immediately for reverse transcription (RT), and the remaining nucleic acids were stored at −20°C.

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**TABLE 1 HIV-1 isolates used from the BBI HIV-1 subtype reference panel**

<table>
<thead>
<tr>
<th>HIV-1 subtype</th>
<th>Strain</th>
<th>Country of origin</th>
<th>NCBI accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>UG275</td>
<td>Uganda</td>
<td>AB485632</td>
</tr>
<tr>
<td>B</td>
<td>BK132</td>
<td>Thailand</td>
<td>AY173951</td>
</tr>
<tr>
<td>C</td>
<td>ZB18</td>
<td>Zambia</td>
<td>AB485641</td>
</tr>
<tr>
<td>D</td>
<td>SE365</td>
<td>Senegal</td>
<td>AB485648</td>
</tr>
<tr>
<td>CRF01_AE</td>
<td>CM240</td>
<td>Thailand</td>
<td>AF067154</td>
</tr>
<tr>
<td>F</td>
<td>BZ126</td>
<td>Brazil</td>
<td>AY173957</td>
</tr>
<tr>
<td>G</td>
<td>BCFDJOUM</td>
<td>Zaire</td>
<td>AB485661</td>
</tr>
<tr>
<td>H</td>
<td>BCPKITA</td>
<td>Zaire</td>
<td>AB485665</td>
</tr>
</tbody>
</table>
TABLE 2 Primer and probe sequences for the HIV-1 virological failure screening assay

<table>
<thead>
<tr>
<th>Primer or probe</th>
<th>Sequence</th>
<th>Function</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMC-forward</td>
<td>5’-TGACCAAGCCACCCGC-3’</td>
<td>Forward primer</td>
<td>EMCV</td>
</tr>
<tr>
<td>EMC-reverse</td>
<td>5’-TAAGATTTGCTTCCCG-3’</td>
<td>Reverse primer</td>
<td>EMCV</td>
</tr>
<tr>
<td>EMC-VIC</td>
<td>5’-TGTTGACCGCATGGTTGCTCC-3’</td>
<td>TAMRA probe</td>
<td>EMCV</td>
</tr>
<tr>
<td>HIV-LTR S4</td>
<td>5’-AACGGCTCAATAAAGCTTGTCATTGA-3’</td>
<td>Forward primer</td>
<td>HXB2 nt 520–543</td>
</tr>
<tr>
<td>3’ UNI-KS-6</td>
<td>5’-GAGGATCTCTAGTATACAGGAGTCAAC-3’</td>
<td>Reverse primer</td>
<td>HXB2 nt 574–600</td>
</tr>
<tr>
<td>HIV-LTR-FAM</td>
<td>5’-TAGTGTGTCGGCCGCTTG-3’</td>
<td>MGR probe</td>
<td>HXB2 nt 554–570</td>
</tr>
</tbody>
</table>

a In the primer and probe designations, EMC stands for encephalomyocarditis virus and LTR stands for the long terminal repeat region of HIV-1.

(ii) Nucleic acid isolation from DBS. At both sites, a preincubation step for DBS was performed. DBS samples were excised by hand using scissors, which were decontaminated between samples with 70% ethanol. For the Nuclisens method, DBS were placed in the provided 2-ml lysis buffer (bioMérieux) in a 9-ml tube. For the QIAamp RNA method (QiaGen GmbH), DBS were placed in 700 µl of the provided buffer, AVL lysis buffer that was aliquoted in 2-ml Eppendorf tubes for use. For both methods, samples were incubated at room temperature with gentle rotation for 30 min, after which filters were removed and NA was isolated as described above for plasma samples.

(iii) Reverse transcription. Purified NA, containing both HIV-1 RNA and IC RNA, was reverse transcribed using the TaqMan real-time PCR system random hexamer RT kit (Life Technologies, Foster City, CA) according to the manufacturer’s instructions. An input of 10 µl NA was used in a final reaction volume of 25 µl. Reactions were carried out according to the following conditions: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. The cDNA generated was used immediately for real-time PCR or stored at 4°C.

(iv) Real-time PCR. HIV-1 and IC cDNA fragments were amplified in the multiplex format. A 25-µl real-time PCR mixture contained 12.5 µl universal TaqMan master mix (Life Technologies), 10 µl cDNA, 300 nM primer EMC-forward (EMC stands for encephalomyocarditis virus), 900 nM primer EMC-reverse, 100 nM tetramethylrhodamine (TAMRA) probe EMC-VIC, 300 nM forward primer LTR S4 (LTR stands for the long terminal repeat region of HIV-1), a mixture of 600 nM HIV-LTR reverse primers 3’ UNI-KS-6 and 3’ UNI-KS-6-AG, and 100 nM minor groove binder (MGB) probe HIV-LTR-FAM (Table 2). The assay was performed at the UMCU, Utrecht, the Netherlands, using an Applied Biosystems 7500 real-time PCR system (ABI7500) (Life Technologies), and in Uganda using a MiniOpticon real-time PCR detection system (Bio-Rad, Hercules, CA). Both systems included a temperature profile allowing for dUTP/uracil-N-glycosylase (UNG) decontamination, namely, 50°C for 2 min; 95°C for 10 min; 45 cycles, with 1 cycle consisting of 95°C for 15 s and 60°C for 1 min. In order to enable run-to-run comparison, a fixed threshold was established for both systems (data not shown).

(v) Assay controls. Positive and negative controls were included in each run. Appropriate performance of the run was judged based on the results of these controls. The positive control consisted of a plasma sample spiked with a fixed concentration of 2.50E+04 copies/ml HIV-1, the threshold cycle (C_T) value acceptance range of which was determined for each real-time system. For this evaluation, the positive-control C_T range was set to 29 to 32. The IC was used to monitor for inhibition of each individual sample. As with the positive control, the C_T value acceptance range of the IC was determined for each real-time system. For this evaluation, the positive-control C_T range was set at 30 to 33. Three negative controls were included, an isolation negative control that consisted of HIV-1-negative human plasma and IC and negative RT and PCR controls that consisted of nuclease-free water and no IC. The result obtained for a sample was considered valid when the positive and IC controls were within their predetermined ranges, and the negative controls were below detection.

Data analysis. A 5-fold serial dilution series of viral RNA for plasma and DBS for all panel subtypes (Table 1) was used to assess dynamic range, level of detection (LOD), and inter- and intra-assay reproducibility. For the ABI7500 (Life Technologies), the serial dilution series ranged from 5.00E+06 to 3.20E+02 copies/ml, and for the MiniOpticon (Bio-Rad), the serial dilution series ranged from 1.00E+06 to 1.60E+03 copies/ml. Linearity was determined and reported as a coefficient of determination (R²) value and slope gradient. Positive-control and IC C_T values were averaged to determine assay precision and reproducibility. Multiple measurements of 5.00E+03 copies/ml dilutions were performed and averaged to establish the VF C_T cutoff range. Theoretically, a one-C_T difference reflects a 2-fold change in target NA in the amplification reaction. The slope of gradient determined using the dilution series gave the number of C_T difference to result in a 1-log-unit change in VL, which were used to interpret an equivalent log10 copies/ml value to assess precision and reproducibility.

For clinical samples, a chi-squared test was performed in order to determine the proportion of virological failures detected using the 5.00E+03 copies/ml cutoff range according to the determined C_T value. A positive result was regarded as a C_T value equal to or lower than the C_T value range designated for 5.00E+03 copies/ml, and a negative result was regarded as a C_T value greater than that. C_T values that were within the 5.00E+03 copies/ml C_T range were considered positive, with a suggestion to repeat in a follow-up test. Samples that were positive or negative in the VFA, but not in the corresponding commercial VL assay were classified as false positive or false negative, respectively. Sensitivity was calculated as the number of true-positive results/(number of true-positive and false-negative results). Specificity was calculated as the number of true-negative results/(number of true-negative and false-positive results).

RESULTS

Assay design. An alignment of the long terminal repeat regions of HIV-1 (LTR) sequences obtained from the Los Alamos database was created representing all HIV-1 reference subtypes (n = 37) and circulating recombinant forms (CRFs) (n = 18). A 145-nucleotide fragment of the HIV-1 5’ long terminal repeat (LTR) R/U5 region was subsequently used for real-time PCR assay design using ABI Primer Express 2.0 software (Life Technologies, CA, USA). Mixed nucleotides were introduced at positions of inter-subtype heterogeneity. The most efficient combinations of designed virological screening failure assay (VFA) primers-probes and a previously published 5’ LTR-based VL primer (17) (Table 2) were optimized and extensively tested in several independent runs with all isolated samples of the subtype panel.

Analytical sensitivity. All HIV-1 subtypes in the panel could be detected with equal efficiency. The assay demonstrated good overall linearity across subtypes, determined by plotting mean C_T values for all subtypes tested. The ABI7500 (Life Technologies)
and the MiniOpticon (Bio-Rad) had coefficients of determination ($R^2$) of 0.996 and 0.975, respectively (Fig. 1). The level of detection (LOD) for both plasma and dried blood spot (DBS) samples was defined by the lowest concentration where no negative VFA result for any subtype was observed in replicates. Results from the serial dilutions determined the LOD for plasma to be 1.00E+03 copies/ml with a mean $C_T$ of 36.65 and SD of 1.12 (data not shown).

Assay precision and reproducibility. To determine precision, the assay was performed by two different operators using positive controls ($n = 12$) with a viral load (VL) of 2.50E+04 copies/ml (4.40 log$_{10}$ copies/ml) in 12 individual runs. Results were highly reproducible for both the HIV-1-positive controls, with a mean VL of 4.17 log$_{10}$ copies/ml and SD of 0.17 log$_{10}$ copies/ml. Intra-assay precision was further assessed in quadruplicate, from isolation to result, for each isolate of the subtype panel, with a VL of 5.00E+03 copies/ml (3.70 log$_{10}$ copies/ml). The mean, SD, and percent coefficient of variation (%CV) of the VL were 3.98 log$_{10}$ copies/ml, 0.24 log$_{10}$ copies/ml, and 8.0%, respectively, for plasma samples, and 3.50 log$_{10}$ copies/ml, 0.33 log$_{10}$ copies/ml, and 13.2%, respectively, for DBS.

Interassay reproducibility was determined at two of the JCRC laboratory sites in Uganda. The assay was performed on 10 high-VL plasma samples with VL of 1.25E+05 to 2.0E+06 copies/ml by 4 different users on different days. The results were highly reproducible with a mean SD of 0.13 log$_{10}$ copies/ml (range, 0.04 to 0.19 log$_{10}$ copies/ml). For all high-VL sample runs ($n = 40$ reactions), the IC results were highly comparable, with a mean SD of 0.11 log$_{10}$ copies/ml (range, 0.03 to 0.23 log$_{10}$ copies/ml).

Accuracy. Three sample sets are shown in Table 3 depicting the qualitative comparison of the VFA and commercial assays using plasma samples. A total of 91.6% (175/191) of the South African samples were accurately classified compared to the commercial assay. Two samples were invalid in the assay, 10 (5.2%) were overestimated (false positive) and six samples (3.1%) were underestimated (false negative) by the assay (Table 2). The sensitivity and specificity were 96.2% and 79.2%, respectively. A total of 92.9% (39/42) of the samples from Tanzania were accurately classified compared to the commercial assay. An additional three samples (7.1%) were overestimated, and no underestimation was observed, with a sensitivity and specificity of 100.0% and 76.9%, respectively.

![FIG 1](http://jcm.asm.org/)

**FIG 1** Linearity of VFA results using two different instruments, ABI7500 and MiniOpticon. The threshold cycle values are shown on the $y$ axis.

### Table 3 Qualitative comparison and method agreement summary of clinical plasma and DBS samples for the VFA

<table>
<thead>
<tr>
<th>Standard assay</th>
<th>Sample standard</th>
<th>Sample VFA</th>
<th>$n^c$</th>
<th>True Pos.</th>
<th>True Neg.</th>
<th>Correctly classified</th>
<th>False Pos.</th>
<th>False Neg.</th>
<th>Incorrectly classified</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan$^e$</td>
<td>Plasma</td>
<td>Plasma</td>
<td>191</td>
<td>153</td>
<td>38</td>
<td>175 (91.6)</td>
<td>10</td>
<td>6</td>
<td>16 (8.4)</td>
<td>96.2</td>
<td>79.2</td>
</tr>
<tr>
<td>Amplicor$^e$</td>
<td>Plasma</td>
<td>Plasma</td>
<td>42</td>
<td>32</td>
<td>10</td>
<td>39 (92.9)</td>
<td>3</td>
<td>0</td>
<td>3 (7.1)</td>
<td>100.0</td>
<td>76.9</td>
</tr>
<tr>
<td>TaqMan$^f$</td>
<td>Plasma</td>
<td>Plasma</td>
<td>176</td>
<td>93</td>
<td>83</td>
<td>169 (96.0)</td>
<td>7</td>
<td>0</td>
<td>7 (4.0)</td>
<td>100.0</td>
<td>92.2</td>
</tr>
<tr>
<td>TaqMan$^e$</td>
<td>Plasma</td>
<td>DBS$^b$</td>
<td>82</td>
<td>62</td>
<td>20</td>
<td>73 (89.0)</td>
<td>4</td>
<td>5</td>
<td>9 (11.0)</td>
<td>92.5</td>
<td>83.3</td>
</tr>
<tr>
<td>VFA$^f$</td>
<td>Plasma</td>
<td>DBS</td>
<td>82</td>
<td>68</td>
<td>14</td>
<td>75 (91.5)</td>
<td>0</td>
<td>7</td>
<td>7 (8.5)</td>
<td>90.7</td>
<td>100.0</td>
</tr>
</tbody>
</table>

$^a$ The results for the virological failure screening assay (VFA) were compared to the results for two commercial assays, Cobas TaqMan system v2 (Roche) (TaqMan) and Cobas Amplicor HIV-1 monitor test v1.5 (Roche) (Amplicor), and a VFA performed in the field.

$^b$ DBS, dried blood spot.

$^c$ n, number of samples tested.

$^d$ True positive (True Pos.), identified by the standard assay as having $\geq 5.00E+03$ copies/ml; true negative (True Neg.), identified by the standard assay as having $<5.00E+03$ copies/ml; false positive (False Pos.), identified by VFA as having $\geq 5.00E+03$ copies/ml and as negative by the standard assay; false negative (False Neg.), identified by VFA as having $<5.00E+03$ copies/ml and identified as positive by the standard assay.

$^e$ The test was performed at UMCU, Utrecht, The Netherlands.

$^f$ The test was performed at JCRC laboratory sites in Uganda.
Results from all the JCRC laboratory sites in Uganda showed a 96.0% (169/176) comparable classification of the VFA performed compared to the commercial assay (Table 3). Seven samples (4.0%) were overestimated, and no underestimation was observed, with a specificity of 100.0% and a sensitivity of 92.2%. In addition, 100.0% of the HIV-1-negative plasma samples (n = 11005) were not detected in the VFA.

Plasma samples versus DBS. Assay linearity and within-run precision were determined for both plasma and DBS samples for subtype A of the subtype panel by plotting mean CT values for the serial dilutions using the ABI7500 at the UMCU, Utrecht, the Netherlands. The slope and R² of standard curves derived from plasma samples and DBS were highly comparable with values of 4.067 and 4.224 and values of 0.951 and 0.971, respectively. On average, the results for plasma samples were 0.32 log₁₀ copies/ml lower than the results for DBS of the same dilution, while the mean SD for plasma was 0.18 versus 0.19 log₁₀ copies/ml for DBS (excluding 2.0E+02 dilution) (Fig. 2).

The performance of the VFA on DBS samples was determined at the JCRC laboratory sites in Uganda. Results were compared with plasma VL results of the same sample that were previously measured with the commercial assay and the VFA. Figure 3 shows a comparison of VFA results for paired plasma and DBS (n = 31) samples given in log copies/ml, determined using a standard curve, compared to plasma VL results generated using TaqMan system v2 (Roche). Above 3.00 log₁₀ copies/ml, it is clear to see comparable qualitative classifications between sample types and assays. In Table 3, there is a summary of qualitative results from all paired plasma and DBS samples. A total of 89.0% (73/82) of the samples compared with the TaqMan were accurately classified. Four samples (4.9%) were overestimated and five samples (6.10%) were underestimated, with a sensitivity and specificity of 92.5% and 83.3%, respectively. The DBS VFA results compared to the plasma VFA results showed a 91.5% (75/82) comparability in classification, with seven samples (8.5%) incorrectly classified, and a sensitivity and specificity of 90.7% and 100.0%. Five of the seven false-negative samples were the same samples that were false negative compared to the TaqMan plasma results, four of which had been stored at room temperature for 40, 70, 184, and 192 days. The remaining three had been stored at −70°C for 330, 455, and 486 days.

DISCUSSION
We have developed and evaluated a qualitative assay to screen for virological failure (VF) during antiretroviral treatment (ART) with particular emphasis on application in resource-limited settings (RLS). The VFA described here can assess ART adherence and inform therapy switching, earlier and with predicted better specificity than clinical and immunological monitoring. Informed therapy switching can prevent unnecessary treatment switching (4) to more expensive and less accessible second-line therapies. Moreover, early detection of VF using the VFA can prevent extended exposure to a failing regimen and possible accumulation of drug resistance mutations that may confer cross-resistance to other drugs or drug classes (8). Using the VFA for early detection of treatment failure could also prevent HIV-1 transmission (18). Viral load (VL) monitoring to determine treatment failure is recommended, including in RLS such as sub-Saharan Africa (19), and should preferably be performed according to the WHO guidelines (6), which suggest targeted use to confirm suspected clinical or immunological failure to prevent unnecessary therapy switching, or earlier use, within 4 to 6 months after ART initiation, to assess adherence and introduce adherence counseling if necessary. The assay presented here meets these WHO requirements and is suitable for use in decentralized settings with less trained medical personnel, compatible with task shifting of ART implementation.

FIG 2 Standard curve of HIV-1 subtype A plasma and DBS samples determined using the ABI7500.

FIG 3 Comparison of viral loads obtained for plasma samples on the TaqMan system v2 (Roche) and for plasma and DBS samples for the same samples using the described VFA method.
The assay was designed as “open platform”: all primer sequences and protocols are openly accessible, and the assay can use various equipment and reagents that can be ordered from multiple manufacturers. This open access and open platform approach increase the affordability and scalability of molecular diagnostics in Africa. Reagent costs for the assay are country dependent, with a per sample cost, based on a run of 10 samples, including controls, of 22.00 U.S. dollars (USD) calculated for UMCU, Utrecht, the Netherlands, and 27.00 USD for JCRC, Uganda. The Southern African Treatment Resistance Network (SATuRN) and ARTA are key supporters of this open movement. SATuRN has negotiated discounted reagents and technical support with Life Technologies in order to decrease the cost and increase access of HIV genotypic drug resistance testing in Africa (http://www.bioafrica.net/saturn/). The VF screening assay described here has the potential to use the same approach. In addition, SATuRN and ARTA have been providing extensive training on the usage of molecular diagnostics for treatment monitoring with more than 1,500 physicians, nurses, and health care workers trained in Africa. These organization training platforms can be used to expand and support the usage of this VF screening assay in Africa.

It is possible to use the VFA in a central laboratory with high-throughput systems such as the Applied Biosystems 7500 real-time PCR system (Life Technologies), but the VFA has also shown remarkable ease of use in smaller, district laboratories with the MiniOpticon real-time PCR detection system (Bio-Rad), as we have shown in Uganda. This is important, as decentralizing VL testing enables faster turnaround times in result reporting to clinicians and their patients, and consequently more efficient treatment monitoring. In addition, a compact real-time thermocycler requires minimal maintenance due to the use of light-emitting diodes (LEDs) instead of xenon lamps and lasers, has no filter wheels, and is easily transportable. However, it has to be emphasized that the assay still requires a laboratory equipped for some molecular diagnostic techniques and staff with medium- to high-level training.

The VFA is designed to control for all steps in the laboratory procedure, ensuring quality and reliability of results. The performance of the assay has demonstrated good correlation to other available VL screening assays in this evaluation and between the two instruments and three Ugandan field sites tested. Evaluation of the VFA for plasma and DBS samples determined the lower limit of detection to be 1.00E+03 and 5.00E+03 copies/ml, respectively. Although the assay was not designed for quantitative application, assay linearity was shown to be adequate, with comparable equations for plasma samples and DBS. The assay demonstrated good intra- and inter assay precision, with highly reproducible results at the 5.00E+03 copies/ml cutoff for plasma samples and DBS. The accuracy of this assay to determine VF showed good correlation with VL results previously determined using commercial VL assays.

The next step to improving access to VL monitoring and reducing associated costs in RLS is routine application with DBS sampling. The use of DBS with commercial and in-house VL assays has already been shown to have some success (20, 21). Preliminary data using spiked whole-blood samples have shown that the current VFA performs well with DBS but with reduced sensitivity compared to plasma samples, which has been previously described (20, 22). Possible reasons for this decreased sensitivity could be due to RNA degradation during storage or loss of sample due to incomplete elution from the filter paper as part of the nucleic acid isolation process. Accuracy of VF determination by commercial assays can also be affected by the DBS method of collection, specifically when blood is either collected directly from finger or heel prick or spotted with a dropper instead of a pipette from EDTA-blood. Unless blood is spotted in exact volumes, it is not possible to determine precise VL using commercial assays. A prospective clinical validation into these collection methods would be needed to investigate their effect on VF determination using the assay described here.

In summary, we have developed a robust and affordable test for VF determination that is open platform and compatible with finger or heel prick DBS collection and pediatric applications and is particularly suitable for application in RLS, such as sub-Saharan Africa. The unique aspect of the assay described here is its multiplex design enabling detection of an internal control in each sample, ensuring accurate and reliable results from isolation to amplification. The VFA could contribute to improved quality of ART and prevention of the development of HIV drug resistance. Further explorations are needed to assess the performance of this test in clinical patient management in African settings. A study along these lines has been performed in Uganda (23).

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