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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Running title: Qualitative HIV-1 virological failure screening assay

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Abstract

Virological failure (VF) has been identified as the earliest, most predictive determinant of HIV-1 antiretroviral treatment (ART) failure. Due to high costs 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 described assay is an internally-controlled qualitative real-time PCR targeting the conserved long terminal repeat domain of HIV-1. This assay was applied with HIV-1 subtypes A-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. The described assay is able to identify VF for all major HIV-1 group-M subtypes with equal specificity, and lower detection limit of 1.00E+03 copies/ml for plasma and 5.00E+03 copies/ml for DBS. Comparative testing yielded accurate VF determination for therapy-switching in 89%-96% of samples compared to gold standards. The assay is robust and flexible, allowing for "open platform" applications and producing comparable results to 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.
Introduction

In 2010 the HIV-1 epidemic was estimated to include 34.0 million (range 31.6-35.2 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 of 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 stock-outs, drug toxicity of “older generation” 1st and 2nd line drugs, failure of patient adherence, drug-drug interactions, lack of qualified healthcare 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 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).
As per the definition of the WHO, VF is a repeated viral load $\geq 5.00\times 10^3$ RNA copies/ml in an individual taking ART for at least four to six months (6). Timely detection of VF by 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 the possibility of VL monitoring and treatment failure is primarily determined using clinical monitoring for stage three and four AIDS-defining illnesses and, if available, immunological monitoring using CD4 counts (6). The inadequacies of CD4 for determining true treatment failure have been described on many occasions (9-12). The clinical-immunological monitoring approach results in individuals being left on sub-optimal 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 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. Secondly, 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, 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, easy to collect and transport, and has proven application for VL testing (13, 14). Thirdly, 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 should be generic, “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 non-government organizations both in Africa and Europe. 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 assay” (VFA) can be readily applied in basic laboratories, using either plasma 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 $\geq 5.00E+03$ copies/ml.

### Materials and Methods

#### Samples

**HIV-1 Subtype Reference panel**

A panel of virus isolates consisting of HIV-1 subtypes A through H (Table 1) was obtained from BBI (BBI Biotech Research Laboratories Inc., Gaithersburg, USA) for assay optimization and evaluation at the University Medical Centre in Utrecht (UMCU), The Netherlands. Serial dilutions were prepared from these stocks using HIV-1 negative human plasma. These dilutions were also used to spike HIV-1 negative whole blood for DBS preparation.
Clinical samples from HIV-1 infected individuals from several African sites were included for further evaluation at the UMCU, the Netherlands. Samples were selected to include several subtypes with a variety of 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+03-3.00E+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® Amplicor 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 6.65E+02-3.07E+05 (median 2.67E+04) copies/ml.

In addition, as part of a technology transfer program, the described assay was applied in three Joint Clinical Research Centre (JCRC) laboratory sites in Uganda, where retrospective plasma samples collected from HIV-1 positive individuals as part of the 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 had been performed in Uganda using the COBAS®AmpliPrep/COBAS®TaqMan®System v2(Roche). A total of 176 plasma samples were included comprising of subtypes A (n=89), D (n=64), and 23 with an unknown subtype, with a VL range of 1.00E+02-1.00E+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 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+01-7.18E+06 (median 2.61E+03) copies/ml. DBS samples were stored at -70°C for 270-515 (median 485) days (n=31), -20°C for 45-112 (median 82) days (n=21), or room temperature for 2-192 (median 126) days (n=30).

Internal Control

An internal control (IC) was added to each clinical sample at a fixed amount of ten percent of the elution volume at the start of nucleic acid isolation. The IC comprised of the non-human RNA virus Encephalomyocarditis virus (EMC) and was prepared at the UMCU, the Netherlands, in batches of single-use aliquots and stored at -80°C until use.

VFA

Nucleic Acid Isolation, Plasma

At the UMCU, 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 100μl plasma was used, or two DBS of 50μl each, and 2.5μl IC. Positive and negative controls were included in each run. Upon completion of the isolation procedure, purified nucleic acids were eluted in 25μl elution buffer.

In Uganda, NA isolation was performed using the QIAamp RNA kit (Qiagen GmbH, Germany), as per manufactures instructions. Input was 100μl of plasma, or two 50 μl DBS, and 5μl IC. Isolated NA was eluted in 50μl elution buffer.
Upon completion of both isolation procedures, the eluates were used immediately for reverse transcription (RT), and remaining nucleic acids stored at -20°C.

**Nucleic Acid Isolation, DBS**

At both sites, a pre-incubation step for DBS was performed. DBS samples where excised by hand using scissors, which were decontaminated between samples with 70% ethanol. For the Nuclisens method, DBS were placed in the provided 2ml lysis buffer (BioMérieux) in a 9ml tube. For the QIAamp RNA method (Qiagen GmbH), DBS were placed in 700ul of the provided Buffer AVL lysis buffer that was aliquoted in 2ml Eppendorf tubes for use. For both methods, samples were incubated at room temperature with gentle rotation for 30 minutes, after which filters were removed and NA isolated as per the plasma samples.

**Reverse Transcription**

Purified NA, containing both HIV-1 RNA and IC RNA, was reverse transcribed using the TaqMan® Real-time PCR system Random Hexamers RT kit (Life Technologies, Foster City, CA) according to manufactures instructions. An input of 10μl NA isolate was used in a final reaction volume of 25μl. Reactions were carried out according to the following conditions: 25°C for 10 minutes, 48°C for 30 minutes and 95°C for 5 minutes. Generated cDNA was used immediately for real-time PCR or stored at 4°C.

**Real-time PCR**

HIV-1 and IC cDNA fragments were amplified in multiplex format. A 25μl real-time PCR reaction contained 12.5μl universal TaqMan® Master Mix (Life Technologies), 10μl cDNA, 300nM primer EMC-forward, 900nM primer EMC-reverse, 100nM probe EMC-VIC, 300nM forward primer LTR S4, a mixture of 600nM HIV-LTR revere primers 3’UNI-KS-6 and
3'UNI-KS-6-AG, and 100nM MGB probe HIV-LTR-FAM (Table 2). The assay was performed at the UMCU, the Netherlands, using an Applied Biosystem 7500 Real-Time PCR System (Life Technologies), and in Uganda using a MiniOpticon™ Real-Time PCR Detection System (BioRad, Hercules, CA). Both systems included a temperature profile allowing for dUTP/UNG decontamination, namely 50°C for 2 minutes; 95°C for 10 minutes; 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. In order to enable run-to-run comparison, a fixed threshold was established for both systems (data not shown).

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 Ct value acceptance range of which was determined for each real-time system. For this evaluation the positive control range was set at Ct 29-32. The IC was used to monitor for inhibition of each individual sample. As with the positive control, the Ct value acceptance range of the IC was determined for each real-time system. For this evaluation the positive control range was set at Ct 30-33. Three negative controls were included, an isolation negative that consisted of HIV-1 negative human plasma and IC, and negative RT and PCR controls which 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

Assay Range

A five-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+06-3.20E+02 copies/ml, and for the MiniOpticon (BioRad) from 1.00E+06-1.6E+03 copies/ml. Linearity was determined and reported as a R^2 value and slope gradient. Positive control and IC Ct 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 Ct cut-off range. Theoretically, a one Ct difference reflects a two-fold change in target NA in the amplification reaction. The slope of gradient determined using the dilution series gave the number of Cts difference to result in a one Log change in VL, which were used to interpret an equivalent Log_{10} 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 cut-off range according to the determined Ct value. A positive result was regarded as a Ct value equal to or lower than the Ct value range designated for 5.00E+03 copies/ml, and a negative result as a Ct value greater than that Ct value range. Ct values that were within the 5.00E+03 copies/ml Ct range were considered positive, with a suggestion to repeat in a follow-up. 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 “true positive/(true positive +false negative)”; specificity was calculated as “true negative/ (true negative + false positive)”.
Results

Assay Design

An alignment of LTR sequences obtained from the Los Alamos database was created representing all HIV-1 reference subtypes (n=37) and Circulating Recombinant Forms (CRF’s; n=18). A 145 nucleotide fragment of the HIV-1 5’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 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 Ct values for all subtypes tested. The ABI7500 (Life Technologies) and the MiniOpticon (BioRad) had co-efficients of determination (R^2) of 0.996 and 0.975, respectively (Figure 1). The level of detection (LOD) for both plasma and 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 LOD for plasma to be 1.00E+03 copies/ml, mean Ct 36.18 and SD 1.19 (data not shown), and for DBS samples to be 5.00E+03 copies/ml mean Ct 36.65 and SD 1.12 (data not shown).

Assay precision and reproducibility

To determine precision, the assay was performed using positive controls (n=12) with a VL
of 2.50E+04 copies/ml (4.40 log\(_{10}\) copies/ml) by two different operators in 12 individual runs. Results were highly reproducible for both the HIV-1 positive controls, mean VL of 4.17 log\(_{10}\) copies/ml with SD of 0.10 log\(_{10}\) copies/ml, and the corresponding IC values, mean VL of 4.14 log\(_{10}\) copies/ml with 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 %CV of the VL were 3.98 log\(_{10}\) copies/ml, 0.24 log\(_{10}\) copies/ml, and 8.0% for plasma, and 3.50 log\(_{10}\) copies/ml, 0.33 log\(_{10}\) copies/ml, and 13.2% for DBS.

Inter-assay reproducibility was determined at two of the JCRC laboratory sites in Uganda. The assay was performed on 10 high VL plasma samples, VL 1.25E+05 - 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 0.04-0.19). 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 0.03-0.23).

**Accuracy**

Three sample sets are shown in Table 3 depicting the qualitative comparison of the VFA and relative commercial assays using plasma samples. A total of 91.6% (175/191) of the South African samples were accurately classified, as compared to the commercial assay. Two samples were invalid in the assay, ten (5.2%) were over-estimated (false positive) and six samples (3.1%) were under-estimated (false negative) by the assay (Table 2). The sensitivity and specificity was 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. A further three samples (7.1%) were over-estimated and no under-estimation was observed, with sensitivity and specificity of 100.0% and 76.9%, respectively.
Results from all the JCRC laboratory sites, Uganda, showed a 96.0% (169/176) comparable classification of the VFA performed as compared to the commercial assay (Table 3). Seven samples (4.0%) were over-estimated and no under-estimation 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=25) were undetectable in the VFA.

Plasma versus DBS

Assay linearity and within-run precision was 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 ABI 7500 at the UMCU, The Netherlands. The slope and $R^2$ of standard curves derived from plasma and DBS were highly comparable, -4.067, -4.224, and 0.951, 0.971, respectively. On average, results for plasma were 0.32 log$_{10}$ copies/ml lower as compared to DBS of the same dilution, while the mean SD for plasma was 0.18 versus 0.19 log$_{10}$ copies/ml for DBS (excluding 2.0E+02 dilution) (Figure 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 log$_{10}$ 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 under-estimated, with
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 (8.5%) being incorrectly classified, and respective 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 VF during ART with particular emphasis on application in RLS. The described VFA 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). 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 four to six months after ART initiation, to assess adherence and introduce adherence counseling if necessary. The presented assay meets these WHO requirements, and is suitable for use in decentralized settings with less trained medical personnel, compatible with task shifting of ART implementation.
The assay was designed as “open platform”: all primer sequences and protocols are open-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 ten samples including controls, of 22.00 USD calculated for UMCU, 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 described VF screening assay 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 over 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 higher-throughput systems such as the Applied Biosystem 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 (BioRad), 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 LED 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 within this evaluation, as well as between the two instruments and three Ugandan field sites tested. The 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 not being designed for quantitative application, assay linearity was shown to be adequate, with comparable equations for plasma and DBS. The assay demonstrated good intra- and inter-assay precision, with highly reproducible results at the 5.00E+03 copies/ml cut-off for plasma and DBS. The accuracy of the described 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 has shown that the current VFA performs well with DBS, however with reduced sensitivity compared to plasma, 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 pre-
cise VL using commercial assays. A prospective clinical validation into these collection methods would be needed to investigate their effect on VF determination using the described assay.

In summary, we have developed a robust and affordable test for VF determination, open platform and compatible with finger- or heel-prick DBS collection also in pediatric applications and particularly suitable for application in RLS, such as sub-Saharan Africa. The unique aspect of the described assay 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).
Acknowledgments

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Figure 1. Linearity of VFA in two different instruments, ABI7500 and MiniOpticon.
Figure 2. Standard curve of HIV-1 subtype A plasma and DBS samples determined using the ABI 7500.

Plasma:  $y = -4.086x + 46.52$
$R^2 = 0.973$

DBS:  $y = -4.210x + 48.14$
$R^2 = 0.972$
Figure 3. Comparison of viral loads obtained for plasma on the TaqMan® System v2 (Roche); and for plasma and DBS for the same samples using the described VFA method.
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<td>HIV-LTR-FAM</td>
<td>5'-TAGTGTGTGCCCAGTCTG-3'</td>
<td>MGB probe</td>
<td>HXB2 nt554-570</td>
</tr>
</tbody>
</table>

EMC: Encephalomyocarditis virus (internal control); LTR: long terminal repeat region of HIV-1; HXB2: HIV-1 reference sequence.
<table>
<thead>
<tr>
<th>Standard Compared</th>
<th>Sample Standard</th>
<th>Sample VFA</th>
<th>n</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* Plasma</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* Plasma</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** Plasma</td>
<td>Plasma</td>
<td>Plasma</td>
<td>176</td>
<td>93</td>
<td>33</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>VFA** Plasma</td>
<td>Dried Blood Spot</td>
<td>Plasma</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>
</tbody>
</table>

Taqman: COBAS®TaqMan® System v2 (Roche); Amplicor: COBAS® Amplicor HIV-1 Monitor test v1.5 (Roche); VFA: Virological Failure assay; DBS: Dried blood spot sample; *: test performed at UMCU, The Netherlands; **: test performed at JCRC, Uganda; n: number of samples tested; True Positive (Pos): Number of samples identified by Standard Compared as being ≥5.00E+03 copies/ml; True Negative (Neg): Number of samples identified by Standard Compared as being <5.00E+03 copies/ml; False Positive (Pos): Number of samples identified by VFA as being ≥5.00E+03 copies/ml, and as being negative by Standard Compared; False Negative (Neg): Number of samples identified by VFA as being <5.00E+03 copies/ml, and as being positive by Standard Compared.