A Partially Multiplexed HIV Drug Resistance (HIVDR) Assay for Monitoring HIVDR Mutations of the Protease, Reverse-Transcriptase (PRRT), and Integrase (INT)

Joshua DeVos,a Kimberly McCarthy,b Victor Sewe,c Grace Akinyi,c Muthoni Junghae,b Valarie Opollo,c Janin Nouhin,d Robert Shafer,d Clement Zeh,a Artur Ramos,a Heather Alexander,a Joy Changa

aInternational Laboratory Branch, Division of Global HIV and Tuberculosis (TB), Center for Global Health, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA
bCDC Kenya, Nairobi, Kenya
cHIV Research Laboratory, Kenya Medical Research Institute-Center for Global Health Research, Kisumu, Kenya
dStanford University, Stanford, California, USA

ABSTRACT As dolutegravir (DTG)-containing HIV regimens are scaled up globally, monitoring for HIV drug resistance (HIVDR) will become increasingly important. We designed a partially multiplexed HIVDR assay using Sanger sequencing technology to monitor HIVDR mutations in the protease, reverse-transcriptase (PRRT), and integrase (INT). A total of 213 clinical and analytical plasma and dried blood spot (DBS) samples were used in the evaluation. The assay detected a wide range of known HIV-1 subtypes and circulating recombinant forms (CRFs) of group M from 139 samples. INT accuracy showed that the average nucleotide (nt) sequence concordance was 99.8% for 75 plasma samples and 99.5% for 11 DBS samples compared with the reference sequences. The PRRT accuracy also demonstrated the average nucleotide sequence concordance was 99.5% for 57 plasma samples and 99.2% for 33 DBS samples. The major PRRT and INT DR mutations of all samples tested were concordant with those of the reference sequences using the Stanford HIV database (db). Amplification sensitivity of samples with viral load (VL) >5000 copies/mL showed plasma exceeded 95% of positivity, and DBS exceeded 90% for PRRT and INT. Samples with VL (1000 to 5000 copies/mL) showed plasma exceeded 90%, and DBS reached 88% positivity for PRRT and INT. Assay precision and reproducibility showed >99% nucleotide sequence concordance in each set of replicates for PRRT and INT. In conclusion, this HIVDR assay met WHO HIVDR assay performance criteria for surveillance, worked for plasma and DBS, used minimal sample volume, was sensitive, and was a potentially cost-effective tool to monitor HIVDR mutations in PRRT and INT.

IMPORTANCE This HIVDR genotyping assay works for both plasma and DBS samples, requires low sample input, and is sensitive. This assay has the potential to be a user-friendly and cost-effective HIVDR assay because of its partially multiplexed design. Application of this genotyping assay will help HIVDR monitoring in HIV high-burdened countries using a DGT-based HIV drug regimen recommended by the U.S. President’s Emergency Plan for AIDS Relief and the WHO.

KEYWORDS drug resistance, human immunodeficiency virus, integrase

In 2014, the Joint United Nations Program on HIV/AIDS (UNAIDS) issued ambitious goals: to achieve 90% of people living with HIV (PLHIV) knowing their HIV status; to have 90% of HIV diagnosed people on antiretroviral therapy (ART); to have 90% of treated PLHIV with their HIV viral load (VL) suppressed by 2020; and to end the HIV/AIDS epidemic as a public health threat by 2030 (1, 2). By 2019, more than 25.4 million adults and children were accessing ART globally (3). With increased PLHIV on ART, the
emergence of HIV drug resistance (HIVDR) is inevitable in populations receiving ART even with appropriate drug regimens and optimal adherence to therapy (4, 5). In 2019, the WHO HIV Drug Resistance Report (5) showed that among 18 low-income and middle-income countries that reported their national HIVDR surveys data to WHO, 12 countries had greater than 10% of populations on first-line ART showed HIVDR to nevirapine (NVP) and efavirenz (EFV), the two most widely used non-nucleoside reverse transcriptase inhibitors (NNRTI) drugs. The reported acquired HIVDR to NNRTI drugs in some countries ranged from 50% to 97%. The acquired dual-class drug resistance to NNRTI and nucleoside reverse transcriptase inhibitors (NRTI) ranged from 21% (Senegal) to 91% (Uganda) (5). In the pediatric population, the prevalence of HIVDR to NVP and EFV before treatment initiation was high, ranging from 34% (Eswatini) to 69% (Malawi). The prevalence of HIVDR to abacavir and lamivudine (the preferred NRTIs drugs for infants) in pretreatment children was also high and exceeded 10% in 4 out of the 9 countries of Malawi, Zimbabwe, South Africa, Togo, Mozambique, Nigeria, Cameroon, Uganda, and Eswatini (5).

The emergence of HIVDR to commonly used NNRTI drugs globally reduces the efficacy of ART for individual patients and public health and may compromise achieving the global targets to end AIDS as a public health threat by 2030 (5). To improve the ART efficacy and overcome the high HIVDR prevalence in the antiretroviral (AVR) regimens, WHO issued new ART guidelines in 2019 to use a dolutegravir (DTG)-based or an integrase inhibitor-based ARV regimen as the preferred first-line and second-line treatments for PLHIV (6).

As the WHO universal policy to use a DTG-based ARV regimen to treat PLHIV approaches full implementation globally (7), a user-friendly, sensitive, cost-effective HIVDR assay to monitor integrase (INT) HIVDR is needed. Currently, there are a few HIVDR kits commercially available to monitor HIVDR for both PRRT and INT. Both Abbott (8) and Advanced Biological Laboratories (ABL) (9) have a single-plex HIV genotyping assay for either PRRT or INT using Sanger technology. Recently, Vela deployed a Federal Drug Administration (FDA)-approved multiplexed HIV genotyping assay, Sentosa, for PRRT and INT genotyping using next-generation sequencing (NGS) (10). All three HIV genotyping assays use plasma and have not demonstrated any assay performance data using DBS as a sample.

Here, we reported the performance data of an HIV-1 genotyping assay that we have reconfigured recently to monitor HIVDR for both PRRT and INT in a partially multiplexed fashion using either plasma or DBS as the sample type. The assay uses Sanger technology and builds upon an in-house HIV-1 genotyping kit for PRRT developed by the Centers for Disease Control and Prevention (CDC) (11) or a user-friendly and cost-effective ABI HIV-1 Genotyping kit (12).

RESULTS

HIV-1 subtype coverage. A total of 139 samples (98 plasma, 41 DBS) with previously known HIV-1 subtypes were tested using the newly configured HIV-1 genotyping assay. The 139 samples included 51 clinical and 88 analytical samples. The PCR positivity of each sample for the PRRT and INT regions was visualized by gel electrophoresis. All HIV-1 subtypes and CRFs of group M included in this study were detected for both PRRT and INT (Table 1).

Accuracy. Table 2 shows the INT accuracy of this genotyping assay. A total of 86 samples were used for the INT accuracy evaluation. Among the 86 samples, 33 were clinical plasma samples and 53 were analytical samples (42 plasma and 11 DBS). Table 3 shows the PRRT accuracy. A total of 90 samples were used, including 26 clinical (16 plasma and 10 DBS) and 64 analytical samples (41 plasma and 23 DBS). Because of the high nucleotide sequence concordance (Tables 2 and 3) at the amino acid level, all major mutations in the reference sequences were successfully identified by the corresponding 90 PRRT (Table 2) and 86 INT (Table 3) samples (data upon request). Table 4 shows the HIVDR profile of three plasma samples which had nucleotide sequence concordance <98% compared to the reference sequences.
Amplification sensitivity. A total of 101 plasma (48 clinical, 53 analytical) and 48 DBS (19 clinical, 29 analytical) samples were included in this performance evaluation. All samples were tested for both PRRT and INT using the reconfigured genotyping assay. The positivity of each sample for the PRRT and INT regions was visualized by electrophoresis using agarose gel and confirmed by sequencing. The amplification sensitivity was analyzed by grouping samples into two groups, one group with VL >5000 copies/mL and the other with VL between 1000 and 5000 copies/mL (Table 5). The amplification status of the samples with negative amplification results is shown in Table 6 (VL >5000 copies/mL; VL 1000 to 5000 copies/mL).

Precision. Table 7 shows the average nucleotide sequence concordance of the 10 pairwise nucleotide sequence comparisons for each of three samples exceeding 99%, respectively. Each pairwise sequence comparison or all sequence comparisons met WHO criteria with over 98% of concordance.

Reproducibility. Table 8 shows the average nucleotide sequence concordance of the 10 pairwise nucleotide sequence comparisons for each of three samples exceeding 99%. Each pairwise sequence comparison or all sequence comparisons met WHO criteria with over 98% of concordance.

**DISCUSSION**

This report aimed to demonstrate performance data of a newly reconfigured HIV-1 genotyping assay to monitor HIVDR mutations for both PRRT and INT in a partially multiplexed genotyping kit. This assay has the potential to be a user-friendly and cost-effective HIVDR assay because it is designed in a partially multiplexed format, which simplifies the assay complexity and procedural steps. Application of this genotyping assay will help to identify mutations associated with HIVDR in HIV high-burdened countries using tenofovir, lamivudine, and dolutegravir (TLD), a DTG-based HIV drug regimen recommended by

**TABLE 1 Subtype coverage**

| Sample subtype | Matrix | Total samples | PRRT POS rate | INT POS rate |
|----------------|--------|---------------|---------------|--------------|
| Subtype A (A1) | DBS    | 2             | 1/2           | 2/2          |
| Subtype A (A1) | Plasma | 4             | 4/4           | 4/4          |
| Subtype B      | DBS    | 16            | 16/16         | 16/16        |
| Subtype B      | Plasma | 53            | 52/53         | 52/53        |
| Subtype C      | DBS    | 9             | 6/9           | 6/9          |
| Subtype C      | Plasma | 21            | 21/21         | 21/21        |
| Subtype D      | DBS    | 1             | 1/1           | 1/1          |
| Subtype D      | Plasma | 4             | 3/4           | 3/4          |
| Subtype F (F1) | DBS    | 1             | 1/1           | 1/1          |
| Subtype F (F1) | Plasma | 5             | 4/5           | 4/5          |
| Subtype G      | DBS    | 3             | 3/3           | 2/3          |
| Subtype G      | Plasma | 1             | 1/1           | 1/1          |
| CRF 01_AE      | DBS    | 2             | 2/2           | 2/2          |
| CRF 01_AE      | Plasma | 1             | 1/1           | 1/1          |
| CRF 02_AG      | DBS    | 3             | 3/3           | 2/3          |
| CRF 02_AG      | Plasma | 6             | 5/6           | 6/6          |
| CRF 06_cpx     | DBS    | 1             | 1/1           | 1/1          |
| CRF 06_cpx     | Plasma | 1             | 1/1           | 1/1          |
| Recombinant of CRF02_AG, A1 | Plasma | 1 | 1/1 | 1/1 |
| Recombinant of B, D | DBS | 1 | 1/1 | 1/1 |
| Recombinant of B, F1 | DBS | 2 | 2/2 | 2/2 |
| Recombinant of D, B | Plasma | 1 | 1/1 | 1/1 |

*POS, positive amplification result.

**TABLE 2 INT accuracy by nucleotide (nt) sequence concordance**

| Sample type | Plasma | DBS |
|-------------|--------|-----|
| No. of samples | 75     | 11  |
| Viral load range (copies/mL) | $10^2$-$10^6$ | $10^1$-$10^4$ |
| % sample $>$98% concordance | 100 (75/75) | 100 (11/11) |
| Average concordance (%) | 99.8 | 99.5 |
the President’s Emergency Plan for AIDS Relief (PEPFAR) and the WHO (6).

Our HIV-1 subtype data showed a wide range of subtype coverage for protease, reverse transcriptase, and integrase (Table 1), which has never been shown in any other HIV genotyping assay, e.g., Abbott ViroSeq HIV-1 Integrase Genotyping kit (13) or Sentosa (10) HIV-1 genotyping assay. Given that the number of samples was limited for each subtype except subtype B, it is impossible in the current evaluation to make a quantitative conclusion about the assay sensitivity of each subtype. It was noticed that three DBS samples previously identified as subtype C, tested negative for both PRRT and INT (Table 1). These three DBS samples had a VL below 5,000 copies/mL which is a challenging VL range for DBS to be amplified. In addition, these three DBS samples were from archived samples stored for 4 to 6 years and may have undergone several freeze-thaw cycles which can compromise the quality of total nucleic acid (TNA). These conditions may explain why the amplification of these samples was unsuccessful in this evaluation (14).

Our INT accuracy demonstrated excellent performance as the average nucleotide sequence concordance was 99.8% for 75 plasma samples and 99.5% for 11 DBS samples compared with the reference sequences (Table 2). The PRRT accuracy also demonstrated great performance as the average nucleotide sequence concordance was 99.5% for 57 plasma samples and 99.2% for 33 DBS samples (Table 3). At the amino acid level, all INT and PRRT samples were concordant with their reference sequences in identifying the major HIVDR mutations as described above in the accuracy results section. The integrase clones from Stanford University showed 100% nucleotide sequence concordance between this assay and the ViroSeq HIV-1 Integrase Genotyping kit. It was noticed that among the 57 PRRT plasma samples (Table 3), three clinical plasma samples showed slightly lower nucleotide sequence concordance at 97.7%, 97.8%, and 97.6%. However, two of them showed the same major DR profiles as the reference sequences (Table 4). The sample (1393001278) with a VL of 1,510 copies/mL picked up an additional NNRTI mutation (V108I) compared to the reference sequence (Table 4). However, it is imprudent to conclude the newly reconfigured genotyping assay is more sensitive than the current ABI HIV-1 genotyping kit for NNRTI mutations because this is only one sample and the sample had low VL.

The amplification sensitivity data showed that both plasma and DBS samples with low and high VL ranges can be successfully amplified for the PRRT and INT regions and meet WHO acceptance criteria. Due to the challenges of obtaining clinical samples, the number

| Sample type | Plasma | DBS |
|-------------|--------|-----|
| No. of samples | 57 | 33 |
| Viral load range (copies/mL) | $10^5-10^6$ | $10^5-10^6$ |
| % sample >98% concordance | 94.6 (54/57)$^a$ | 100 (33/33) |
| Average concordance (%) | 99.5 | 99.2 |

$^a$Three clinical samples with 97.6%, 97.7%, and 97.8% of nucleotide sequence concordance.
of plasma samples with VL ranging between 1000 to 5000 copies/mL (Table 5), and DBS samples in both high and low VL ranges were limited (Table 5). Another challenge of the samples in this evaluation was that some archived samples had up to 4 to 10 years of storage with multiple freeze-thaw cycles previously. These conditions were reflected in the amplification results of five samples with VL >5000 copies/mL and six samples with VL ranging from 1000 to 5000 copies/mL (Table 6), which showed negative amplification results for either PRRT or INT or both (Table 6).

The precision and reproducibility data of the reconfigured assay demonstrated excellent performance using fresh plasma samples. The sequences of all replicates per sample in both precision and reproducibility data showed >99% of average nucleotide sequence concordance among five replicates of each sample for both PRRT and INT (Table 7 and 8). We noticed that samples with a higher VL demonstrated a higher average of nucleotide sequence concordance compared to samples with a lower VL for both PRRT and INT.

For the HIVDR assay application, the most valuable feature of this HIVDR assay is that both plasma and DBS samples may be used for HIVDR genotyping. The Abbott ViroSeq HIV-1 Integrase Genotyping kit (8), ABL DeepCheck (9), and Sentosa (10) HIV-1 genotyping assay have not demonstrated data on the performance of DBS. The DBS application of this assay is very important in resource-limited settings or remote areas where plasma samples may not be feasible to collect or transport for VL testing and genotyping (15–17). It is even more important in pediatric populations where a collection of DBS samples from infants or younger children for genotyping is often performed. One of the user-friendly features of this genotyping assay is that it has the flexibility to use TNA extracted from other sample extraction procedures, e.g., QIAamp Viral RNA Mini (18). This is supported by our recent quality control for molecular diagnostics (QCMD) HIVDR proficiency testing results showing that our integrase sequence generated using Nuclisens easyMag and this genotyping assay was 100% concordant with the consensus nucleotide sequence (data upon request). In other words, our integrase result is concordant with the integrase sequence results from

### Table 5: Amplification sensitivity of samples

| No. of samples | Sample type | PRRT POS<sup>a</sup> rate (%) | INT POS<sup>a</sup> rate (%) | Sample nature (clinical/analytical) |
|----------------|-------------|-------------------------------|-----------------------------|-----------------------------------|
| VL >5000 copies/mL | | | | |
| 23 | DBS | 95.7 (22/23) | 91.3 (21/23) | 15/8 |
| 78 | Plasma | 97.4 (76/78) | 97.4 (76/78) | 34/44 |
| VL 1000-5000 copies/mL | | | | |
| 25 | DBS | 88.0 (22/25) | 88.0 (22/25) | 4/21 |
| 23 | Plasma | 91.3 (21/23) | 91.3 (21/23) | 14/9 |

<sup>a</sup>POS = positive amplification result.

### Table 6: Samples with amplification failure

| Sample ID | Sample type | VL (copies/mL) | Sample nature | PRRT PCR | INT PCR |
|-----------|-------------|----------------|---------------|----------|---------|
| VL >5000 copies/mL | | | | | |
| 2009696248 | DBS | 6400 | Clinical | --<sup>a</sup> | + |
| 2009696256 | DBS | 7400 | Clinical | -- | + |
| 2009695657 | DBS | 11600 | Clinical | + | -- |
| 3026002458 | Plasma | 9100 | Clinical | -- | -- |
| 2010693484 | Plasma | 15350 | Unknown | -- | -- |
| VL 1000-5000 copies/mL | | | | | |
| D000012498 | Plasma | 1470 | Clinical | -- | + |
| BD90007998041117DD | Plasma | 2724 | Clinical | + | -- |
| 3005055807 | Plasma | 5032 | Unknown | -- | -- |
| 3005055104 | DBS | 1573 | Unknown | -- | -- |
| 3005050272 | DBS | 2400 | Unknown | -- | -- |
| 3005050270 | DBS | 3547 | Unknown | -- | -- |

<sup>a</sup> --, negative amplification result; +, positive amplification result.
other submitters using Qiagen as the extraction method and other integrase genotyping assays (data upon request). This assay also has the flexibility to use different models of ABI sequencers, e.g., ABI 3130, ABI 3500, and ABI 3730. The ABL genotyping kit may have the flexibility to use different sample extraction procedures and different sequencing instruments but the Abbott ViroSeq HIV-1 Integrase Genotyping kit and Sentosa only use their formulated sample extraction procedures. In the case of Sentosa, laboratories will be required to purchase the entire Vela automated system from sample extraction to sequence data analysis to perform the Sentosa genotyping assay, which may not be cost-effective in resource-limited countries.

Another important feature of this genotyping assay is the low sample input requirement and relatively shorter testing procedures. Because of our assay design, 10 μL of 25 μL of TNA from 200 μL of plasma or one DBS spot may be used to perform HIVDR genotyping for both PRRT and INT which saves sample and workflow. This contrasts with the ViroSeq HIV-1 Integrase Genotyping kit and ABL, which both require 500 μL of plasma for PRRT genotyping and an additional 500 μL for INT genotyping. Sentosa is a great multiplexed and fully automated HIVDR assay using NGS technology. However, it requires 730 μL of plasma for the sample input (10). The sample saving feature of our HIVDR genotyping assay is particularly helpful to countries using remnant HIV VL samples to monitor HIVDR mutations as part of TLD implementation and scaleup as recommended by WHO (19) and PEPFAR. It is even more helpful to the pediatric HIVDR monitoring program, where low volume plasma samples or DBS are often collected for HIVDR genotyping.

Our results should be interpreted considering the following limitations. As VL copies decrease (<1000 copies/mL), the amplification success rate also decreases (20). This is a common observation with HIVDR assays, including the Abbott ViroSeq HIV-1 Integrase Genotyping kit and Sentosa (10, 13). This limitation makes HIVDR mutation monitoring for patients with low-level viremia (200 to 999 copies/mL) challenging. While significantly increasing the sample volume may improve performance, this is often not practical, especially in resource-limited countries. Another limitation of this study is the sample size, especially the number of DBS samples tested and analyzed. DBS sample testing was limited because of patient consent issues. It was also difficult to access clinical samples, especially unsuppressed patients on a DTG-based regimen due to the high rate (94%) of VL suppressed patients on the DTG regimen (21).

In conclusion, this partially multiplexed HIV-1 genotyping assay meets all WHO HIVDR assay performance criteria for HIVDR monitoring. It works for both plasma and DBS, uses a minimum amount of sample, is sensitive, easy to use, and has the potential to be a low-cost assay to monitor HIVDR mutations in PRRT and INT regions.

### MATERIALS AND METHODS

**Samples.** A total of 213 samples with VL ranging from $10^2$ to $10^6$ copies/mL were used in the performance evaluation of this HIV-1 genotyping assay to assess subtype coverage, accuracy, amplification

| TABLE 7 Precision |
|-------------------|
| VL (copies/mL)   | Sample ID          | Average of replicate concordance |
|                  |                    | PRRT (%) | INT (%)       |
| 123,000          | 1358100795P1       | 99.5      | 99.7          |
| 15,000           | 13902200104P       | 99.1      | 99.4          |
| 5,030            | 4168P3             | 99.0      | 99.2          |

| TABLE 8 Reproducibility |
|-------------------------|
| VL (copies/mL) | Sample ID  | Average of replicate concordance |
|               |            | PRRT (%) | INT (%) |
| 175,000       | 14133000001R3 | 99.4      | 99.9     |
| 17,200        | 1339200329R2  | 99.3      | 99.4     |
| 2,135         | 1408601627R1  | 99.0      | 99.3     |
sensitivity, precision, and reproducibility. Among the 213 samples, 125 were remnant clinical or patient samples from the African region, e.g., the HIV VL monitoring program in Kenya or from previous HIVDR external quality assessment programs (EQAs), e.g., Virology Quality Assurance (VQA) Program (22) or quality control for molecular diagnostics (QCMD) (23), and 88 were analytical or cultured viral isolates among which 82 were from VQA and QCMD and 6 INT mutation-specific isolates were donated by Stanford University. Because the 213 samples varied by type (plasma or DBS), source, fresh or archived, and volume, the number of samples used in each assay performance characteristic was not the same.

**Sample extraction.** Before HIVDR testing, samples were extracted using the NucliSENS easyMag by the International Laboratory Branch (ILB) of the Division of Global HIV and TB (DGHT), CDC, or NucliSENS miniMag at the Kenya Medical Research Institute/Center for Global Health Research (KEMRI/CGHR) HIV research laboratory, Kisumu, Kenya, following manufacturer’s instructions (bioMérieux SA, F-69280 Marcy l’Etoile, France) (24). The extraction procedures yielded 25 μL of total nucleic acids (TNA) from either 200 μL of plasma or one DBS spot with 75 μL of whole blood. Of the 25 μL of TNA, 10 μL was used for the genotyping assay (Fig. 1A and B).

**HIVDR assay configuration.** CDC reconfigured the assay design and workflow based on the CDC in-house assay (11) or ABI HIV-1 genotyping kit for PRRT only (12) (Fig. 1A).

Fig. 1B shows the reconfigured genotyping assay with the capacity to monitor HIVDR for both PRRT and INT. The INT component of this reconfigured assay covers the entire integrase region and generates an amplicon of 1045 bp long for INT sequencing (Fig. 1B). Similar to the current ABI HIV-1 genotyping kit, the reconfigured assay is designed to introduce 10 μL out of 25 μL of TNA from 200 μL of plasma or one DBS spot into the reverse transcription-polymerase cycling reaction (RT-PCR) to coamplify two ~1 KB fragments, PRRT and INT, in the same reaction. These two amplicons are then amplified by nested PCR and sequenced separately (Fig. 1B). The reconfigured HIV-1 genotyping assay was assembled into a kit format by Thermo Fisher Scientific before our assay performance evaluation. The kit was manufactured under ISO 13485 compliance. To make the kit user-friendly, the kit is configured in two modules, one for amplification and the other for sequencing. Each kit tests up to 48 samples. Each module provides a ready-to-use master mix, enzyme, and positive and negative controls for amplification or sequencing, respectively (Fig. 1B). We were not able to share the INT primer sequences in this report for proprietary reasons. The details of thermal cycling profiles of RT-PCR, nested-PCR, and sequencing reactions can be found in the kit user guide on the Thermo Fisher Scientific website (12) after January 2022.

**Sample testing and sequence analysis.** Testing was performed by CDC with support from the KEMRI/CGHR HIV research laboratory. Both sites used the reconfigured genotyping kit assembled by Thermo Fisher Scientific. CDC performed all data analysis using ReCall (25) and BioEdit 7.2 (26) sequence editing software. Sequence editing and quality assurance checks before HIVDR profile analysis were performed using the Stanford HIVdb program, version 8.8 (27).

The accuracy of the newly configured genotyping assay was evaluated by comparing INT and PRRT sequences with their reference sequences. The source of the reference sequences was based on the sample source. For clinical samples, the sequence of the same sample from the ViroSeq HIV-1 Integrase genotyping kit or previous ABI HIV-1 genotyping assay was used as the reference sequence. These sequences were analyzed using the HIVdb program, version 8.8 (27). For EQA remnant samples, the consensus sequences from the EQA exercises were used as the reference sequences (22, 23). Finally, for the INT-specific isolates from Stanford University, the sequences with INT DR mutations from Stanford University were used as the reference sequences. The accuracy performance was determined using WHO acceptance criteria for HIVDR assay validation, ≥90% of nucleotide sequence comparisons between the test result and the reference must be at least 98% identical with nonmatching mixtures counted as a difference (28).
The amplification sensitivity was assessed using WHO HIVDR assay validation criteria. Plasma samples with VL $>5000$ copies/mL need to present $90\%$ of amplification positivity. For DBS samples with a VL of $>5000$ copies/mL, amplification positivity should exceed $90\%$, and for DBS with VL between 1000 and 5000 copies/mL amplification positivity should exceed $50\%$ (28). The assay precision was evaluated using three fresh plasma samples with VLs of 123,000, 15,000, and 5,030 copies/mL from the Kenya HIV VL monitoring program. Each sample had five replicates. All three samples along with their replicates were extracted and tested together at the same time by a laboratory scientist. Using WHO acceptance criteria, $90\%$ (9 of 10) of pairwise comparisons for each sample must be at least $98\%$ identical (with nonmatching mixtures counted as a difference).

The assay reproducibility was evaluated using three fresh plasma samples with VLs of 175,000, 17,200, and 133 copies/mL from the Kenya HIV VL monitoring program. Each sample had five replicates that were run in separate test batches. Each batch was extracted and tested on different days by two laboratory scientists (28). The WHO acceptance criteria for reproducibility are the same as the precision (re: $\geq 90\%$ (9 of 10)) of pairwise comparisons for each sample must be at least $98\%$ identical (with nonmatching mixtures counted as a difference).

Ethics review. The use of the residual plasma samples from the Kenya routine HIV VL monitoring program was approved by KEMRI/CGH Scientific and Ethics Review Unit (SERU) in Kenya. The residual plasma samples were delinked from patient identifier information before testing and data analysis. This project was reviewed in accordance with CDC human research protection procedures and was determined to be research not involving human subjects or identifiable biospecimens.

ACKNOWLEDGMENTS

We thank Chunfu Yang for her leadership in the development of the CDC in-house HIV-1 genotyping test for protease, reverse-transcriptase (PRRT) in 2011, which is the base of the current partially multiplexed HIVDR assay for PRRT and integrase. We sincerely thank Thermo Fisher Scientific, USA, especially Karen Clyde, Edgar Schreiber, and Joshua Trotta, for their efforts to put this assay into a kit format before our assay evaluation.

The study was reviewed in accordance with the U.S. Centers for Disease Control and Prevention (CDC) human research protection procedures and was determined to be research not involving human subjects or identifiable biospecimens.

This research was supported by the President’s Emergency Plan for AIDS Relief (PEPFAR) through HHS/CDC Head Quarter funding and partially supported by a CDC grant under the mechanism of 6 NU2GGH001972-04-02. The findings and conclusions in this paper are those of the authors and do not necessarily represent the official position of the funding agencies.

We declare a conflict of interest. Joshua DeVos is a co-inventor in U.S. patent US100537418B2 while Robert Shafer has received research funding from Vela Diagnostics and InSilixa Inc.

REFERENCES

1. UNAIDS. 2014. 90-90-90 An ambitious treatment target to help end the AIDS epidemic. https://www.unaids.org/sites/default/files/media_asset/90-90-90_en.pdf.
2. UNAIDS. 2014. Fast-Track - Ending the AIDS epidemic by 2030. https://www.unaids.org/en/resources/documents/2014/JC2686_WAD2014report.
3. UNAIDS. 2020. Global HIV & AIDS statistics - 2020 fact sheet. https://www.unaids.org/en/resources/fact-sheet.
4. WHO. 2017. HIV drug resistance report 2017. https://www.who.int/publications/i/item/9789241512831.
5. WHO. 2019. HIV drug resistance report 2019. https://www.who.int/publications/i/item/WHO-CDS-HIV-19.21.
6. WHO. 2019. Update of recommendations on first- and second-line antiretroviral regimens. Policy brief. https://www.who.int/publications/i/item/WHO-CDS-HIV-19.15.
7. Doherty M. 2020. Introduction and overview of WHO diagnostics, treatment and care priorities. 23rd International AIDS Conference.
8. Seattìa KK, Choga WT, Mogwele M, Diphoko T, Maruapula D, Mupfumi L, Musonda RM, Rowley CF, Avolas A, Kasovske I, Moyo S, Gaseitsiwe S. 2019. Comparison of an in-house ‘home-brew’ and commercial ViroSeq Integrase genotyping assays in HIV-1 subtype C samples. PLoS One 14:e024292. https://doi.org/10.1371/journal.pone.024292.
9. ABL BW. 2015. Advanced Biological Laboratories (ABL) launches HIV and HCV DeepChek SingleRound genotyping and sequencing assays. https://www.businesswire.com/news/home/20151106005788/en/Advanced-Biological-Laboratories-ABL-Launches-HIV-and-HCV-DeepChek%C2%AE-SingleRound-Genotyping-and-Sequencing-Assays.
10. VELA. 2019. Package insert, Sentosa SQ HIV-1 genotyping reagents. https://www.fda.gov/media/132293/download.
11. Zhou Z, Wagar N, DeVos JR, Rottinghaus E, Diallo K, Nguyen DB, Bassey O, Ugbera R, Wadonda-Kabondo N, McConnell MS, Zulu I, Chilima B, Nkengasong J, Yang C. 2011. Optimization of a low cost and broadly sensitive genotyping assay for HIV-1 drug resistance surveillance and monitoring in resource-limited settings. PLoS One 6:e28184. https://doi.org/10.1371/journal.pone.0028184.
12. Thermo Fisher Scientific. 2021. Genotyping of HIV-1 to detect drug resistance. https://www.thermofisher.com/us/en/home/life-science/sequencing/sanger-sequencing/applications/genotyping-hiv-detect-drug-resistance.html.
13. Wallis CL, Viana RV, Saravanan S, Silva de Jesus C, Zeh C, Halvas EK, Mellors JW. 2017. Performance of Celera RUO integrase resistance assay across multiple HIV-1 subtypes. J Virol Methods 241:41–45. https://doi.org/10.1016/j.jviromet.2016.12.008.
14. Inzaule S, Yang C, Kasembeli A, Nafisa L, Okonji J, Oyaro B, Lando R, Mills LA, Larsonson K, Thomas T, Nkengasong J, Zeh C. 2013. Field evaluation of a broadly sensitive HIV-1 in-house genotyping assay for use with both plasma and dried blood spot specimens in a resource-limited country. J Clin Microbiol 51:529–539. https://doi.org/10.1128/JCM.02347-12.
15. Rodriguez-Auad JP, Rojas-Montes O, Maldonado-Rodriguez A, Alvarez-Munoz MT, Munoz O, Torres-Ibarra R, Vazquez-Rosales G, Lira R. 2015. Use of dried plasma spots for HIV-1 viral load determination and drug resistance genotyping in mexican patients. Biomed Res Int 2015:240407. https://doi.org/10.1155/2015/240407.

16. Rottinghaus EK, Ugbenia R, Diallo K, Bassey O, Azeez A, Devos J, Zhang G, Aberle-Grasse J, Nkengasong J, Yang C. 2012. Dried blood spot specimens are a suitable alternative sample type for HIV-1 viral load measurement and drug resistance genotyping in patients receiving first-line antiretroviral therapy. Clin Infect Dis 54:1187–1195. https://doi.org/10.1093/cid/cis015.

17. Zhang G, DeVos J, Medina-Moreno S, Wagar N, Diallo K, Beard RS, Zheng DP, Mwachari C, Riwa C, Jullu B, Wangari NE, Kibona MS, Ng’Ang AL, Raizes E, Yang C. 2018. Utilization of dried blood spot specimens can expedite nationwide surveillance of HIV drug resistance in resource-limited settings. PLoS One 13:e0203296. https://doi.org/10.1371/journal.pone.0203296.

18. Qiagen. 2020. QIAamp viral RNA mini handbook.

19. WHO. 2021. Laboratory-based survey of acquired HIV drug resistance using remnant viral load specimens. WHO, Geneva, Switzerland.

20. Santoro MM, Fabeni L, Armenia D, Alterci C, Di Pinto D, Forbici F, Bertoli A, Di Carlo D, Gori C, Carta S, Fedele V, D’Arrigo R, Berno G, Ammassari A, Pinnetti C, Nicastri E, Latini A, Tommasi C, Boumis E, Petrossllo N, D’Offizi G, Andreoni M, Ceccherini-Silberstein F, Antinori A, Perno CF. 2014. Reliability and clinical relevance of the HIV-1 drug resistance test in patients with low viremia levels. Clin Infect Dis 58:1156–1164. https://doi.org/10.1093/cid/ciu020.

21. Nabita VM, Navaggi P, Campbell J, Conroy J, Harwell J, Magambo K, Middlecote C, Caldwell B, Katureebe C, Namuswenye N, Atugonza R, Musoke A, Musinguzi J. 2020. High acceptability and viral suppression of patients on Dolutegravir-based first-line regimens in pilot sites in Uganda: a mixed-methods prospective cohort study. PLoS One 15:e0232419. https://doi.org/10.1371/journal.pone.0232419.

22. NIH. 2021. NIH. Duke virology quality assurance (VQA), HIV-1 drug resistance sequencing participation requirements and scoring procedures. https://www.hanc.info/content/dam/hanc/documents/laboratory/vqa-resources/VQA_HIV-1_GEN_DR_PT_Information%20v20_20211101.pdf.

23. Pandit A, Mackay WG, Steel C, van Loon AM, Schuurman R. 2008. HIV-1 drug resistance genotyping quality assessment: results of the ENVA7 Genotyping Proficiency Programme. J Clin Virol 43:401–406. https://doi.org/10.1016/j.jcv.2008.08.021.

24. Biomerieux. 2005. Nuclisens easymag. https://www.biomerieuxindia.in/product/nuclisens-easymag.

25. Woods Cjb CK, Liu TF, Chui CKS, Chu AL, Wynhoven B, Hall TA, Trevino C, Shafer RW, Harrigana PR. 2012. Automating HIV drug resistance genotyping with RECall, a freely accessible sequence analysis tool. J Clin Microbiol 50.

26. Hall T. 2008. BioEdit. https://thallscience.github.io/.

27. Stanford University. 2008. HIV drug resistance database. https://hivdb.stanford.edu/.

28. WHO. 2020. WHO HIVResNet HIV drug resistance laboratory operational framework. https://www.who.int/publications/i/item/978-92-4-000987-5.