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RESEARCH ARTICLE

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

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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

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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) regimen, 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.

TABLE 1 Subtype coverage

Sample subtype	Matrix	Total samples	PRRT POS[a] 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

[a]POS, positive amplification result.

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 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	10^3-10^4
% sample >98% concordance	100 (75/75)	100 (11/11)
Average concordance (%)	99.8	99.5

TABLE 3 PRRT accuracy by nucleotide (nt) sequence concordance

Sample type	Plasma	DBS
No. of samples	57	33
Viral load range (copies/mL)	10^3-10^6	10^2-10^4
% 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.

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

TABLE 4 PRRT HIVDR mutation profiles of the three plasma samples with nucleotide sequence concordance <98%[a]

Sequence Name	PR Start	PR End	RT Start	RT End	Subtype (%)	Pcnt Mix	PR Major	NRTI	NNRTI	% nt concordance	Viral Load (cps/ml)
1377003609.v2	6	99	1	251	A (3.57%)	2.2	None	None	A98AG	97.7	1,670
1377003609.v1	6	99	1	251	A (5.02%)	0	None	None	A98G		
1392001278.v2	6	99	1	251	A (4.73%)	0	None	None	V108I,E138A	97.8	1,510
1392001278.v1	6	99	1	251	A (4.73%)	0	None	None	E138A		
1666201767.v2	6	99	1	251	D (5.60%)	2.1	None	M184V	K103N,V106VI,V108I	97.6	34,400
1666201767.v1	6	99	1	251	D (6.86%)	0	None	M184V	K103N,V106I,V108I		

[a]nt, nucleotide; PR, protease; RT, reverse-transcriptase; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; v1, reference sequence; v2 = testing sequence. The red font is to indicate the difference between test sequence and reference sequence.

TABLE 5 Amplification sensitivity of samples

No. of samples	Sample type	PRRT POS[a] rate (%)	INT POS 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

[a]POS = positive amplification result.

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 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	−[a]	+
2009696256	DBS	7400	Clinical	−	+
2009696567	DBS	11600	Clinical	+	−
3026002458	Plasma	9100	Clinical	−	−
2010693484	Plasma	15350	Unknown	−	−
VL 1000-5000 copies/mL					
D000012498	Plasma	1470	Clinical	−	+
BD900079978041117DD	Plasma	2724	Clinical	+	−
3005055807	Plasma	5032	Unknown	−	−
3005055104	DBS	1573	Unknown	−	−
3005050272	DBS	2400	Unknown	−	−
3005050270	DBS	3547	Unknown	−	−

[a]−, negative amplification result; +, positive amplification result.

TABLE 7 Precision

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

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 (8, 9) 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 8 Reproducibility

		Average of replicate concordance	
VL (copies/mL)	Sample ID	PRRT (%)	INT (%)
175,000	1413300001R3	99.4	99.9
17,200	1359200329R2	99.3	99.4
2,135	14086.01627R1	99.0	99.3

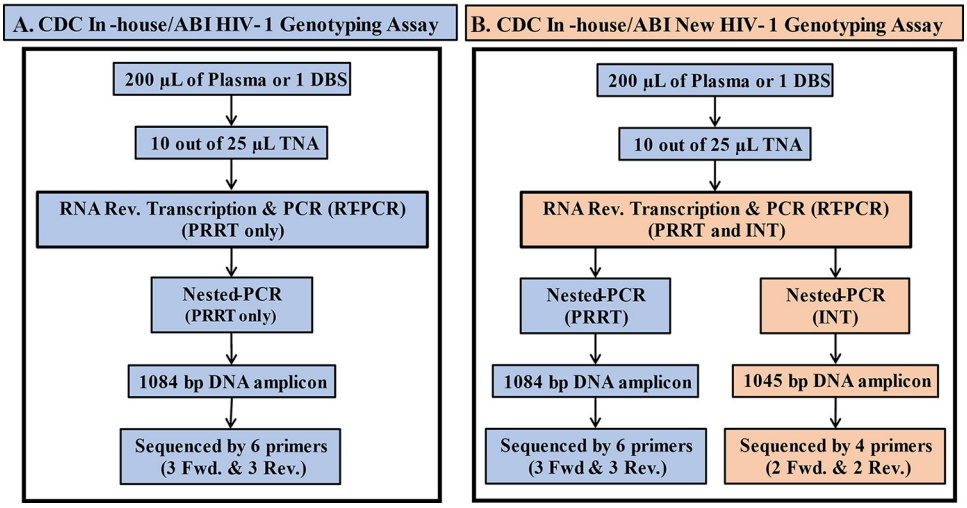

FIG 1 (A) CDC in-house/ABI HIV-1 genotyping assay. (B) CDC in-house/ABI new HIV-1 genotyping assay.

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 (EQA), 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 a multiplexed 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 ≥95% of amplification positivity and plasma samples with VL between 1000 and 5000 copies/mL need to reach ≥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 2,135 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: ≥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/CGHR 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.

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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 nor identifiable biospecimens.

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We declare a conflict of interest. Joshua DeVos is a co-inventor in U.S. patent US10053741B2 while Robert Shafer has received research funding from Vela Diagnostics and InSilixa Inc.

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