Performance characteristics of a modified HIV-1 drug resistance genotyping method for use in resource-limited settings [version 1; peer review: 2 approved]

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Abstract

Background: HIV-1 drug resistance (HIVDR) assays are critical components of HIV clinical management programs in the face of emerging drug resistance. However, the high costs associated with existing commercial HIVDR assays prohibit their routine usage in resource-limited settings. We present the performance characteristics of a modified commercial HIVDR testing assay.

Methods: A total of 26 plasma samples were used to validate and assess the accuracy, precision, reproducibility and amplification sensitivity of a modified HIVDR assay by HIV genotyping. In addition, a cost comparison between the original and the modified assay was performed using the ingredient costing approach.

Results: The performance characteristics of the modified assay were in agreement with the original assay. Accuracy, precision and reproducibility showed nucleotide sequence identity of 98.5% (confidence interval (CI), 97.9–99.1%), 98.67% (CI, 98.1–99.23) and 98.7% (CI, 98.1–99.3), respectively. There was no difference in the type of mutations detected by the two assays ($\chi^2 = 2.36, p = 0.26$). Precision and reproducibility showed significant mutation agreement between replicates (kappa = 0.79 and 0.78), respectively ($p < 0.05$). The amplification sensitivity of the modified assay was 100% and 62.5% for viremia ≥1000 copies/ml and <1000 copies/ml respectively. Our assay modification translates to a 39.2% reduction in the cost of reagents.

Conclusions: Our findings underscore the potential of modifying commercially available HIVDR testing assays into cost-effective, yet accurate assays for use in resource-limited settings.
Introduction

There has been an unprecedented increase in support for HIV diagnosis, treatment and monitoring programmes. Various multinational groups including the U.S. President’s Emergency Plan for AIDS Relief (PEPFAR) and Global Fund have increased funding of HIV management programs leading to improved access to antiretroviral therapy (ART)\textsuperscript{1,2}. The outcome of the expanded access to ART is significant decline of HIV/AIDS-associated morbidity and mortality\textsuperscript{2}. As a result, there has been an increase in both life expectancy and duration for patients on lifelong ART translating to high risk of HIV drug resistance (HIVDR) development. To sustain the success achieved following improved ART coverage, continuous clinical, virologic and immunological monitoring of patients on ART is required to ensure these positive treatment outcomes are maintained\textsuperscript{3,4}. Unfortunately, development and transmission of HIV drug resistance threatens to derail these achievements\textsuperscript{5}. HIV drug resistance testing (DRT) is routinely used for clinical care in high-income countries; however, it is not available to a majority of patients in resource-limited settings due to the high costs of implementation and limited trained manpower.

The World Health Organization (WHO) launched the Global Action Plan (GAP) against HIVDR for resource-limited countries whereby two of the proposed approaches included continuous innovation and capacity building of laboratory staff\textsuperscript{6}. Scientists have attempted to develop alternative affordable methods for HIV drug resistance monitoring to improve access\textsuperscript{7-10}. One such test was developed by Centre for Disease Control and Prevention (CDC) and is currently distributed by Thermo Fisher Scientific\textsuperscript{11}, and is herein referred to as the original assay. The assay is used to genotype the genetically diverse HIV-1 virus from plasma samples to detect resistance mutations in the protease and reverse transcriptase genes with a good subtype inclusivity rate compared to other commercial DRT assays in the market\textsuperscript{11}.

In the present study, we modified this commercial HIV DRT protocol by a 50% reduction of reagent volumes for nested PCR and cycle sequencing reactions for genotyping of HIV-1 drug resistance. The performance characteristics of the modified assay were assessed and evaluated for suitability and reliability using WHO guidelines for assay validation. In addition, cost comparison was performed to determine the cost implication of the assay modification.

Methods

Samples

A total of 26 blood samples were collected in EDTA tubes from patients attending Kenyatta National Hospital HIV clinic laboratory for routine viral load monitoring. Plasma was separated by centrifugation at 2000g for 10 minutes. The plasma samples were stored at -80°C freezer (Nuve, Ankara, Turkey). Remnant plasma samples were used for this study.

Ethical approval

Ethical clearance for the study was obtained from University of Nairobi-Kenyatta National Hospital Ethics Review Committee (UoN-KNH-ERC). The need for consent was waived since remnant plasma from HIV viral load testing were used in this study.

Viral RNA extraction

HIV-1 RNA was extracted from 500 µl plasma using the PureLink\textsuperscript{TM} extraction kit according to manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Briefly, 25 µl proteinase K and 500 µl plasma were transferred into 2-ml microcentrifuge tube. Lysis buffer (500 µl) was added to the mixture, vortexed for 15 seconds, and incubated at 56°C for 15 minutes. Next, 500 µl of 96% ethanol was added to the reaction tube, and vortexed for 15 seconds and incubated at room temperature for 5 minutes. The lysate was transferred to sterile viral spin column, washed twice with 500 µl of wash buffer and finally eluted in 40 µl of elution buffer. RNA was stored at -80°C. The extraction procedure was similar for both the original and modified method.

HIV DRT

Original genotyping method. The Thermo Fisher Scientific\textsuperscript{TM} HIV Genotyping workflow that amplifies a 1.1-kb fragment covering codons 6–99 of the protease gene and codons 1–251 of the reverse transcriptase (RT) gene was implemented according to manufacturer’s instructions\textsuperscript{6}. Briefly, reverse transcriptase PCR and nested PCR were performed using the HIV-1 Genotyping Kit: Amplification Module. Primers used are shown in Table 1. Cycle sequencing was performed using the HIV-1 Genotyping Kit: Cycle Sequencing Module. The resulting cycle sequencing products were analyzed using an ABI 3730 genetic analyzer. The consensus sequences were generated using ReCall (requires free registration) and drug resistance mutations were interpreted using the Stanford HIVdb genotyping resistance interpretation algorithm.

| Primer Sequence (5’–3’) | Step | Fragment size |
|-------------------------|------|--------------|
| 1 F1a, 5'-TGAAAGAAGTGYACTGARAGRCAGGCTAAT | RT-PCR | 2057 to 2085 |
| 2 F1b, 5'-A5TCGARAGRCAGGCTAATT TAG | RT-PCR | 2068 to 2092 |
| 3 R, 5'-CTCCCTGCATAAATCTGACTTG | RT-PCR | 3370 to 3348 |
| 4 PRT-F2, 5'-CTTTARCTTCCCTCARATCCTCT | NESTED-PCR | 2243 to 2266 |
| 5 RT-R2, 5'-CTCTGTATGTCATTGACAGTCC | NESTED-PCR | 3326 to 3304 |
Modified genotyping system Specific reaction steps of the original protocol were modified by reducing the reagent volumes by half.

The HIV Genotyping kit: Amplification module was used for RT-PCR according to the manufacturer’s instructions. Briefly, 10 µl RNA was denatured by incubating at 65°C for 10 minutes and added to 40 µl RT-PCR Master Mix containing SuperScript™ III One-Step RT-PCR with Platinum™ Taq High Fidelity Enzyme. The reaction conditions for RT-PCR were 50°C for 45 minutes where first-strand cDNA synthesis was performed. Enzyme inactivation and denaturation of cDNA-RNA hybrid was accomplished by incubating the reaction at 94°C for 2 minutes. Second-strand synthesis and PCR amplification was carried out in 40 cycles of 94°C for 15 seconds, 50°C for 20 seconds, 72°C for 2 minutes and a final extension for 10 minutes at 72°C using Veriti thermocycler.

Nested PCR reaction mix was prepared by adding 2 µl RT-PCR product to 23.7 µl of nested mastermix containing 0.12 mM of each of inner primers, 1X GeneAmp Gold Buffer II, 2 mM MgCl₂, 400 mM each dNTP and 0.25 µl AmpliTaq Gold™ LD DNA Polymerase enzyme (Applied Biosystem, CA, USA). The target pol region was amplified using Veriti Thermocycler in a final reaction volume of 25.7 µl (Applied Biosystems, CA, USA). The reaction conditions were 94°C for 4 cycles, 40 cycles of 94°C for 15 seconds, 55°C for 20 seconds, 72°C for 2 minutes and a final extension at 72°C for 10 minutes. Nested PCR products of 1.1 kb were confirmed by gel electrophoresis. A 5 µl aliquot of nested PCR product was added to 4 µl ExoSAP-IT™ PCR Purification Reagent and incubated at 37°C for 15 min and 80°C for 15 minutes in a Veriti thermocycler.

Cycle sequencing was performed using the HIV Genotyping kit: Cycle Sequencing module. Six overlapping primers, labelled F1, F2, F3, R1, R2, and R3 were used. One microliter nested PCR product was added to 9 µl of each cycle sequencing mix. The cycle sequencing reaction conditions were 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes.

The Big Dye XTerminator purification kit was used to purify the sequencing reaction by adding 10 µl of the Big Dye XTerminator and 45 µl SAM solution to cycle sequencing products. The reaction plate was vortexed at 1,800 rpm for 30 minutes. The plate was then centrifuged at 1000g for 2 minutes at room temperature. Next, 30 µl of purified cycle sequencing products were transferred to a reaction plate and analyzed using an ABI 3730 genetic analyzer (Applied Biosystem, CA, USA). Sequences were generated using ReCall and drug resistance mutations interpreted using the Stanford HIVdb genotyping resistance interpretation algorithm (output files available as Extended data15) and the International AIDS Society (IAS) 2011 mutation list13.

Modified assay validation Performance characteristics of the modified assay were assessed using the WHO/HIV ResNet guidelines, including accuracy, precision, reproducibility and amplification sensitivity14.

Accuracy. Accuracy refers to the agreement between a result and an expected reference value. In genotyping assays, nucleotide sequence identity is used. Ten samples were analyzed using both methods and the degree of concordance in mutations identified was compared based on the 2017 IAS mutations list11. Nucleotide sequence identity between the paired sequences was assessed using the EMBOSS pairwise alignment tool. The WHO recommends 90% nucleotide sequence similarity as the cutoff point for assay performance characteristics14.

Precision. This is the ability of an assay to generate the same result on multiple replicates of the same sample within a test run. Three samples were analyzed using the modified method in n=4 replicates. The degree of concordance of detected mutations within replicates was determined. Nucleotide sequence identity was also determined using the EMBOSS program for pairwise alignment14.

Reproducibility. This is the ability of a test to produce the same result on multiple aliquots of the same sample in different test runs. Ten samples were analyzed in duplicates using the modified method on different days. Nucleotide sequence identity of sequences obtained was assessed using EMBOSS program for pairwise alignment14.

Amplification sensitivity. Amplification sensitivity is defined as the percentage of successful genotyping tests amongst specimens with a specific viral load range. In this study, sixteen samples with viral loads ranging between 207 and 86,040 copies/ml were analyzed using the modified assay to determine the viral load ranges at which ≥95% of the samples were successfully genotyped14.

Reagent cost comparison An ingredient costing approach was utilized to estimate reagent costs for both original and modified assays at RNA extraction, DNA/RNA amplification, gel electrophoresis, and sequencing steps. All costs were converted to US dollar using 2019 conversion rate.

Statistical analyses Quantitative variables were expressed as mean ± standard deviations (SD). The McNemar test was used to assess significance in the discordant mutations between the modified and the original assay. Precision and reproducibility were assessed using the Cohen kappa statistic. Wilcoxon signed-rank test was used to compare the original and modified assays in base calling for mixed bases between the two methods. Statistical Package for Social Sciences (SPSS) version 22 (IBM, NY, USA) was used for all data analysis.

Results Validation of the modified HIV-1 drug resistance genotyping assay Accuracy of sequence identity and mutation detection. The mean nucleotide identity was 98.5% (confidence interval (CI), 97.92–99.1%). A total of 68 mutations were detected, including 9 (13%) mutations in the protease gene and 59 (87%) in the
reverse transcriptase (RT) gene as shown in Table 2. The original assay detected 67 drug resistance mutations, including 2 mutations (V106I and K225H) which were missed by the modified assay. On the other hand, the modified assay detected 68 drug resistance mutations, including four mutations (D67DG, K101KPQT, K70KN and I50IL) which were not detected by the original method. A total of six mutations were discordant (V106I, P225H, D67DN, I50IL, K101KPQT, and K70KN). Of the six discordant drug resistance mutations, two were completely discordant (V106I and P225H) while four (D67DN, K101KPQT, K70KN and I50IL) were partially discordant. Three of the discordant mutations (D67DN, K101KPQT, and K70KN) were detected as mixtures in the modified assay as non-mixtures (D67N, K101P and K70N) in the original assay and as shown in Table 3. A high mutation concordance was obtained between the two assays at $\chi^2 = 2.36, p = 0.26$. The significance of mixture base-calling between the two methods was determined using the Wilcoxon signed rank test, which showed no significant difference in mixtures detected by the two methods at $p = 0.089$.

**Precision and reproducibility of the modified assay.** Assessment to examine the precision (intra-assay precision) and reproducibility (inter-assay precision) of the modified assay were performed by analyzing $n=3$ samples in quadruplicate in a single test run for precision. The mean nucleotide sequence identity within the replicates was 98.67% (CI, 98.1–99.3). Reproducibility was assessed by testing 10 samples in duplicate on different days using the modified assay. The mean nucleotide sequence identity was 98.6% (CI, 98.2–99.0). The overall agreement of drug resistance mutations detected by precision and reproducibility was significant at kappa value of 0.792 and 0.778, respectively, as shown in Table 4.

**Amplification sensitivity of the modified assay.** A total of 16 samples with a median viral load of 832.5 copies/ml (IQR 403.5–2454.25) were used to assess amplification sensitivity. The modified assay showed an amplification sensitivity of 100% for samples with viral load $\geq 1000$ copies/ml and 62.5% for samples with viral load <1000 copies/ml as shown in Table 5.

**Comparison of costs of reagents between the original and modified assays**

After a 50% reduction in reagent volumes in the amplification and sequencing reactions, we compared the cost between the original and the modified method. The cost of analyzing one sample from extraction to sequencing was decreased from $97 to $59. This represents about 39.2% cost reduction as shown in Table 6.

### Table 2. Summary of the paired sequence comparisons performed by the original and the modified assay.

| Basis of comparison | Original vs. Modified |
|---------------------|----------------------|
| Number of samples   | 10 vs. 10            |
| Nucleotide identity | 98.5% (CI, 97.9–99.1%) |
| Number of mutations detected | 67 vs. 68 |
| Number of discordant mutations | 6 (2$^a$ vs. 4$^b$) |

$^a$Discordant mutations detected by the original assay.  
$^b$Discordant mutations detected by the modified assay.

### Table 3. Pairwise comparison of drug resistance mutations identified by the original assay with those identified by the modified assay.

| ID | Reverse transcriptase gene | | Protease gene |
|----|---------------------------|-----------------------------|-------------|
|    | Original                  | Modified                    | Original   | Modified |
| 1  | M184V, H221Y              | M41L, M184V, H221Y          |            |
| 2  |                          |                             |            |
| 3  | V106VM, V179E, Y181C, Y188YC, A62V, V75I, K219KE | A62V, V75I, D67DN$^b$, K219KE, V106VM, V179E, Y181C, Y188YC | I50IL$^b$ |
| 4  | M41L, M184V, L210W, T215FY, A98G, V106I, Y188L | M184V, L210W, T215FY, Y188L, K238KT | M46I, I84V | M46MI, I84V |
| 5  | M184V, K103N, V108I, P225H$^b$ | M184V, K103N, V108I | | |
| 6  | K65R, Y115F, M184V, V106M, G190A | K65R, Y115F, M184V, V106M, G190A | | |
| 7  | D67N, K70R, L74I, M184V, K219Q, A98G, K103N, V108I, E138Q, V179L, K238T, L74V | D67N, K70R, L74I, M184V, K219Q, A98G, K103N, V108I, E138Q, V179L, K238T, L74V | | |
| 8  | K219QK, Y181C, Y188L, H221Y, M41L | K47R, Y181C, Y188L, H221Y | | |
| 9  | D67N, K70R, M184V, T215F, K219E, A98G, V108V, Y181C, Y181Y | D67N, T215F, K219E, A98G, K101KPQT$^b$, E138Q, Y181YC, K238N | M46I, I54IV, V82A | M46I, I54IV, V82A |
| 10 | D67DG, K70N, M184V, K101P, E138Q | D67DG, K70KN$^b$, M184V, T215F, K219E, A98G, K101P, Y181YC | M46MI, I54IV, V82VA | M46MI, I54IV, V82VA |

$^a$Discordant drug resistance associated mutations detected in original assay.  
$^b$Discordant drug resistance associated mutations detected in the modified assay.
| Parameter          | Number of samples | Replicates | Kappa value | P value | Nucleotide identity |
|-------------------|-------------------|------------|-------------|---------|---------------------|
| Precision         | 3                 | 12         | 0.792       | 0.58    | 98.67%              |
| Reproducibility   | 10                | 20         | 0.778       | 0.4     | 98.6%               |

Table 5. Viral load levels and HIV-1 subtypes detected in plasma samples used for amplification sensitivity testing.

| Sample ID | VL (Copies/ml) | Subtype |
|-----------|----------------|---------|
| 1         | 86,040         | A1      |
| 2         | 12,155         | A1      |
| 3         | 2,802          | D       |
| 4         | 2,700          | D       |
| 5         | 1,717          | A1      |
| 6         | 1,547          | A1      |
| 7         | 1,100          | C       |
| 8         | 1,040          | A1      |
| 9         | 625            | D       |
| 10        | 612            |         |
| 11        | 514            | C       |
| 12        | 459            |         |
| 13        | 385            | A1      |
| 14        | 312            | A1      |
| 15        | 214            | A1      |
| 16        | 207            |         |

Table 6. Cost comparison at several steps of the procedure of HIV drug resistance testing.

| Reaction steps       | Original assay, $ | Modified assay, $ |
|----------------------|-------------------|-------------------|
| RNA extraction       | 4.2               | 4.2               |
| DNA/RNA amplification| 37.2              | 19.2              |
| Gel electrophoresis  | 1.2               | 1.2               |
| Sequencing           | 54.4              | 34.4              |
| Total cost           | 97                | 59                |

Discussion

Here, we show that the results of the modified assay are comparable to those produced by the original assay in the performance characteristics analyzed including accuracy, precision, reproducibility and amplification sensitivity. We observed a high concordance of drug resistance mutations detected by both original and modified assays. When testing for accuracy, we observed high nucleotide sequence similarity (98.5±0.94%) versus original assay. In addition, we reported 98.67% (CI, 98.1–99.3) sequence similarity when assessing precision and 98.6% (CI, 98.2–99.0) when assessing reproducibility of the modified assay15. Furthermore, the modified assay showed 100% sensitivity for VL >1000 copies/ml which is the recommended indicator for HIV treatment failure16. In general, the modified assay met all the requirements in the WHO Genotyping assay validation recommendations. Altogether our findings demonstrate that reducing the volumes of reagents in the original assay by up to half results in a low-cost HIV drug resistance assay that could be utilized in resource-limited settings without compromising the quality of testing.

Accuracy was assessed by analyzing 10 samples with viral load ranges (4258 –78924 copies per ml) by both the original and the modified assays and all 10 samples were successfully amplified and genotyped with high nucleotide sequence identity (98.5±0.94%). All the clinically relevant mutations were reproducible by both methods in spite of some minor discordance between the two methods. The WHO recommends a minimum of 90% sequence similarity for genotyping assays14. Our findings of 98.5±0.94% nucleotide sequence similarity are in agreement with those reported by Zhou et al. who reported 99.41%. In addition, analysis of mutation concordance between the original and the modified assay showed high mutation concordance also reported by Inzaule et al. Our findings for mutation concordance (93%) were lower than reported by Manasa et al. (100%). The modified assay precision assessment showed a high degree of nucleotide sequence identity within replicates. Similar results for precision (98.22%) were reported by Zhou et al. High sequence similarity was also obtained for reproducibility of the modified assay. Our findings for reproducibility (98.6%) were similar to those reported by Zhou et al., who obtained (98.94%). Amplification sensitivity was defined as the percentage of samples successfully genotyped at a given plasma viral load range. In this study, all samples with viral load ≥1000 copies/ml were successfully genotyped, a finding that was similar to that reported by a previous study8. For samples with plasma viral loads <1000 copies/ml, our modified assay successfully genotyped 62.5% of the samples which is slightly below 63.6% reported by a previous study8. The WHO criteria for HIVDR testing requires that patients with
persistent viral load \( \geq 1000 \text{ copies/ml} \) to be tested for HIV drug resistance\(^7\). Our assay was 100% efficient in genotyping these samples. Furthermore, with 62.5% amplification sensitivity for low level viremia (LLV) samples, highlighted the possibility of using the modified assay in genotyping patients with LLV. However, there is need to perform further large-scale studies to clearly establish the performance of the modified method in LLV patients.

We detected six discordant mutations in both original and modified assays. Out of the six discordant mutations, four were mixed-base mutations, which were detected exclusively by the modified assay. The differential detection of discordant mutations between the two assays could be attributed to detection of nucleotide mixtures as a result of subjectivity in basecalling or amplification bias\(^{18,19}\). Previous studies have also reported a significant contribution of nucleotide mixtures to discrepancies of mutations between Viroseq and an in-house assay\(^6\,^7\).

The comparison of cost between the two assays revealed a reduction of HIV drug resistance testing cost by 39.2% per test. These findings suggest that access to HIV drug resistance services in resource-limited settings could be increased through innovative modifications of existing commercially available assays. A small sample size was used because this was a small modification of a validated test already in use. The applicability of this assay can be demonstrated further by testing a larger number of samples. One limitation of the study is the samples used were from patients failing first line treatment and not exposed to protease inhibitors leading to under representation of drug resistant mutations in the protease gene.

**Conclusion**

Our findings underscore the potential utility of a modified cost-effective HIV-1 drug resistance assay for testing plasma samples in resource-limited settings. The performance characteristics of the modified assay were satisfactory and therefore the cheaper assay could be one of the approaches of increasing access to HIVDR tests.

**Data availability**

**Underlying data**

GenBank accession numbers for HIV-1 pol and gag sequences isolated from each participant using each technique are available in Table 7.

**Extended data**

Figshare: Performance Characteristics of a Modified HIV-1 Drug Resistance Genotyping Method for use in Resource Limited Settings: https://doi.org/10.6084/m9.figshare.9114833\(^1\).

This study contains the following extended data:

- Accuracy testing data (Stanford HIV Drug Resistance reports; PDF)
- Precision testing data (Stanford HIV Drug Resistance reports; PDF)
- Reproducibility testing data (Stanford HIV Drug Resistance reports; PDF)
- EM_Accuracy_Precision_Repro_AmpSensitivity (summary of HIV Drug Resistance Mutations detected; xlsx)
- Gel images (TIF)

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

**Grant information**

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**Table 7. Accession numbers of HIV-1 sequences generated in this study.**

| HIV-1 sequence isolate | GenBank accession number | Assay type |
|------------------------|--------------------------|------------|
| 19BI03                 | MN240769                 | Original   |
| 19GB05                 | MN240770                 | Original   |
| 19KE01                 | MN240771                 | Original   |
| 19KE02                 | MN240772                 | Original   |
| 19KE06                 | MN240773                 | Original   |
| 19KE07                 | MN240774                 | Original   |
| 19KE08                 | MN240775                 | Original   |
| 19KE09                 | MN240776                 | Original   |
| 19KE10                 | MN240777                 | Original   |
| 19UG04                 | MN240777                 | Original   |
| BI03                   | MN240777                 | Modified   |
| GB05                   | MN240780                 | Modified   |
| KE01                   | MN240781                 | Modified   |
| KE012                  | MN240782                 | Precision run |
| KE014                  | MN240783                 | Precision run |
| KE02                   | MN240784                 | Modified   |
| KE06                   | MN240785                 | Modified   |
| KE07                   | MN240786                 | Modified   |
| KE08                   | MN240787                 | Modified   |
| KE09                   | MN240788                 | Modified   |
| KE10                   | MN240789                 | Modified   |
| SE011                  | MN240790                 | Modified   |
| UG013                  | MN240791                 | Precision run |
| UG04                   | MN240792                 | Modified   |
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This is an innovative approach to reducing costs of offering HIV drug resistance testing in resource-limited settings. The authors have shown a thorough understanding of the literature in HIV drug resistance testing and implementation in resource-limited settings. They have related their findings to the majority of publications that presenting performance characteristics of in-house HIV drug resistance tests. It would be interesting for the authors to compare the performance of the ‘modified assay’ with the Viroseq assay as well.

The authors followed the World Health Organization (WHO) recommendations for validating 'in-house' HIV drug resistance assays and clearly showed that the results of the modified assay were comparable to the commercial assay. Furthermore, the authors have demonstrated that these assay modifications resulted in a significant decrease in cost of implementation. I however wonder why the authors did not present results for reducing reaction volumes for the RT-PCR step. It is conceivable that they may have at least attempted and therefore should comment if the results did not meet the WHO criterion for assay validation.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes
Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** HIV drug resistance and molecular genomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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Reviewer Report 18 September 2019

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**Title**
1. Ok.

**Abstract**
1. ‘We present the performance characteristics of a modified commercial HIVDR testing assay.’ Should be changed to ‘this study established the performance characteristics of a modified commercial HIVDR testing assay’.

2. The abstract lacks a recommendation.

3. The key word ‘assay validation’ is missing in the abstract.

**Introduction**
1. ‘Various multinational groups’ use ‘agencies’ instead of ‘groups’.

2. ‘…leading to improved access to antiretroviral therapy (ART)’ replace with ‘leading to improved access to HIV clinical care’. (HIV care is not limited to antiretroviral therapy only).

3. ‘…the outcome of the expanded access to ART is significant decline of HIV/AIDS associated morbidity and mortality.’ Replace with ‘…the outcome of the expanded access to clinical care has been a significant drop in HIV/AIDS related morbidity and mortality.’

4. ‘As a result, there has been an increase in both life expectancy and duration for patients on lifelong ART translating to high risk of HIV drug resistance (HIVDR) development.’ This statement has not been brought out clearly & what aspect of ‘duration’ is being implied in this case?’
5. ‘……transmission of HIV drug resistance’ add ‘strains’ after resistance.

6. ‘One such test was developed by Centre for Disease Control and Prevention (CDC) and is currently distributed by Thermo Fisher Scientific10, and is herein referred to as the original assay’. Change to ‘One such test, herein referred to as the original assay, was developed by the Centre for Disease Control and Prevention (CDC) and is currently distributed by Thermo Fisher Scientific.’

**Methods**

**Samples**
1. Are 26 samples enough to validate an assay? And how did you arrive upon this sample size?
2. How were the samples collected? By venipuncture or cardiac puncture? Be very clear by which method.
3. After clinic, delete the word ‘laboratory’. Patients attend clinics, not clinic laboratories.
4. -80 is already a freezing condition. Delete the word ‘freezer’, the statement will still make sense without the subtle repetition.
5. What are ‘remnant plasma samples’? Were they the ones stored in the freezer? What defines them as ‘remnant’?

**Ethical approval**
1. It is claimed here that consent was waived, yet in the sample collection, it is clearly stated that the samples were collected from patients. How and why was the consent waived, and by whom?

**Viral RNA extraction**
1. Add ‘the’ before manufacturer’s.
2. Microcentrifuge ‘tubes’. Plural.

**HIV DRT**
1. As this was a study validating an assay that relied heavily on reagent volumes, why aren’t the volumes of the original assay and their reaction temperatures not included here?

**Reagent cost comparison**
1. As the year is not out yet and conversion rates keep fluctuating, which conversion rate did you use?

**Provide references for definition of the following words:** Precision, Reproducibility, Accuracy and amplification sensitivity

**Statistics**
1. Significance was set at what $p$ value?

**Results**
1. Only two drug resistance genes are targeted in this assay, what informed the decision to arrive on these two sets of genes?
2. Table 5 should be the one introducing the results subtopic. Introduce the samples, the viral loads before the validation assay results are presented.
3. Remove the grids from the tables.

**Discussion**
1. Use third person singular when discussing your results.

2. You don’t ‘show’ in discussion what has been shown in the results section.

3. Delete ‘altogether’ in the first paragraph.

4. What did Zhou et al.\(^1\) find out, and which procedure were they modifying? Include the year of the study too. As it has been used as the main reference, is it the only study that supports your findings? Are there studies that contradict your findings, and if there are, why aren’t they included here?

5. If the samples were obtained from patients who had not been exposed to protease inhibitors, why did your study focus on protease inhibitors?

References
1. Zhou Z, Wagar N, DeVos JR, Rottinghaus E, Diallo K, Nguyen DB, Bassey O, Ugbena R, Wadonda-Kabondo N, McConnell MS, Zulu I, Chilima B, Nkengasong J, Yang C: Optimization of a low cost and broadly sensitive genotyping assay for HIV-1 drug resistance surveillance and monitoring in resource-limited settings.\textit{PLoS One}. 2011; 6 (11): e28184 PubMed Abstract I Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature? Yes

Is the study design appropriate and is the work technically sound? Yes

Are sufficient details of methods and analysis provided to allow replication by others? Yes

If applicable, is the statistical analysis and its interpretation appropriate? I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility? Partly

Are the conclusions drawn adequately supported by the results? Yes

\textbf{Competing Interests:} No competing interests were disclosed.

\textbf{Reviewer Expertise:} Microbial and vaccine immunology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
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