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Protocols

# Performance of an in-house multiplex PCR assay for HIV-1 drug resistance testing – A cheaper alternative

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ABSTRACT

*Background:* Currently, most HIV drug resistance PCR assays amplify the protease-reverse transcriptase (PR-RT) fragment separately from the integrase (IN) fragment. The aim of this study was to develop a multiplex PCR assay that simultaneously amplifies PR-RT and IN fragments for HIV-1 drug-resistance testing.
*Methods:* The in-house multiplex PCR assay was evaluated on extracted total nucleic acids obtained from the National Health Laboratory Service (NHLS) and Lancet laboratories. Sanger sequencing was performed on amplicons, and HIV-1 drug-resistance mutations (DRMs) were assessed using HIV Stanford drug resistance database.
*Results:* This study tested 59 patient samples with known HIV-1 viral load and DRM results; 41 from Lancet and 18 from NHLS. In-house multiplex PCR assay detected one or both fragments in most samples but had higher sensitivity for detection of IN fragment (93.2 %) compared to PR-RT fragment (83.1 %). There was 100 % concordance between Lancet assay versus in-house assay sequence data for IN DRMs, but lower concordance with PR-RT (87.0 %). The in-house multiplex PCR assay's precision and reproducibility analysis showed ≥99.9 % sequence similarity and yielded similar DRM results for both PR-RT and IN fragments.
*Conclusions:* The in-house multiplex PCR assay demonstrated satisfactory performance and higher sensitivity for IN fragment amplification. This could be a cost-effective method for HIV-1 drug resistance testing as both PR-RT and IN fragments are successfully amplified in one reaction in most samples.

## 1. Background

South Africa has the highest number of HIV-1 infections globally with 7.8 million people living with HIV (PLWH) by the end of 2022, and has the largest antiretroviral therapy (ART) programme globally (UNAIDS, 2024; HSRC, 2023). The UNAIDS 95–95–95 fast-track goals aim to end the HIV/AIDS pandemic by 2030 and requires that 95 % of PLWH should know their status, 95 % of those who are aware of their status should be on ART, and 95 % of those who are on ART should have full viral load (VL) suppression (UNAIDS, 2023). South Africa achieved 90–91–94 by the end of 2022 (HSRC, 2023). Poor VL suppression creates opportunities for the emergence of drug-resistant mutations, which limits the effectiveness of the ART regimen. The major risk factor for emergence of drug resistance is low plasma levels of antiretroviral (ARV) drugs mainly due to poor adherence to treatment (Emamzadeh-Fard et al., 2012; Gómez et al., 2022; Conway, 2007). The current South African treatment guidelines include dolutegravir (DTG) for use in adult and paediatric patients who are newly diagnosed with HIV-1 infection, and in those failing an NNRTI-based or protease inhibitor (PI)-based regimen (SADH, 2023).

DTG is available in a fixed dose combination together with tenofovir disoproxil fumarate (TDF) and lamivudine (3TC) for treatment of HIV-1-infected adults, and is commonly abbreviated as TLD (SADH, 2023). Virological failure is defined as a VL >1000 copies/ml on at least two occasions over the course of two years whilst on a PI/DTG-containing regimen with adherence >80 %. According to the local ART

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guidelines, patients with virological failure should be referred to an expert who will advise on ARV drug resistance testing and changing to a new ART regimen (SADH, 2023).

Currently, most HIV-1 drug resistance assays amplify the PR-RT fragment separately from the IN fragment (Rhee et al., 2016; Obasa et al., 2020; Gupta et al., 2017; Bareng et al., 2022; Cunningham et al., 2001; Aghokeng et al., 2011). The aim of this study was to develop a multiplex PCR assay that co-amplifies PR-RT and IN fragments in one reaction in order to simplify HIV-1 drug-resistance testing.

## 2. Methods

### 2.1. Study design and population

This was a retrospective study that used plasma samples with known HIV-1 VL to evaluate the performance of an in-house multiplex PCR assay that simultaneously amplifies PR-RT and IN fragments. The samples were obtained from a public sector laboratory, National Health Laboratory Service (NHLS); and from a private sector laboratory, Lancet diagnostic laboratory. Both these laboratories are based in the Gauteng province and are accredited by the South African National Accreditation System (SANAS). Residual HIV-1 VL plasma samples with no ARV resistance data were obtained from the NHLS laboratory, and extracted total nucleic acid samples with known VL and DRM data were obtained from the Lancet laboratory. This study was approved by the University of Pretoria, Faculty of Health Sciences Research Ethics Committee (Reference number – 282/2020) and also by NHLS and Lancet laboratories.

### 2.2. Total nucleic acid (NA) extraction

Total NAs were extracted from NHLS plasma samples using the EMAG automated extraction system (BioMérieux, Marcy-l'Étoile, France). A plasma input volume of 500 μL was used and NAs were eluted in a volume of 60 μL and stored at −70 ºC. The eluates obtained from the Lancet laboratory were extracted using the NucliSENS easyMAG instrument (BioMérieux, Marcy-l'Étoile, France), stored at −70ºC and sent to our laboratory on dry ice.

### 2.3. Amplification and sequencing

In-house PCR primers were used for the PR-RT fragment and published primers (Onoriode Digban et al., 2020) were used for the IN fragment (Supplementary Table 1). Details of the reagents and thermocycling conditions are summarised in Supplementary Table 2.

Initially, the PR-RT and IN primers were evaluated in separate nested PCR reactions to assess if they could amplify their target fragments. This initial optimisation informed the design of the IN-F1 primer used with published IN primers. The PR-RT and IN primers were later evaluated in a multiplex PCR protocol. A four-fold serial dilution of a sample with known VL was performed to assess the detection limit of the in-house multiplex PCR assay, followed by assessment of the assay's performance on low VL samples of <2000 copies/ml.

Sanger sequencing of PCR amplicons was performed commercially (Inqaba Biotechnical Industries, Pretoria, South Africa) in four overlapping regions to ensure bidirectional sequencing for each fragment (Supplementary Table 1). Sequences were edited in CLC Main Workbench 2.1 software (Qiagen, Hilden, Germany) and consensus sequences were generated and viewed in BioEdit 7.2.5, (https://bioedit.software.informer.com/download/). Sequence alignment with reference sequences was performed using the online version of the MAFFT program (https://maft.cbrc.jp/alignment/server/). Phylogenetic analysis was performed using MEGA software (https://www.megasoftware.net/), and neighbour-joining trees were drawn with a 1000 bootstrap replicates. DRMs were identified using the Stanford HIV drug resistance database (https://hivdb.stanford.edu/hivdb/by-sequences/). Precision analysis was performed in five samples that were amplified in duplicate within the same PCR experiment and then sequenced for both PR-RT and IN fragments. For reproducibility analysis, six samples were amplified in two different PCR experiments at different time points, and also sequenced for both PR-RT and IN fragments at different time points. Consensus sequences obtained from samples used for precision and reproducibility analysis were aligned in MAFFT program, followed by pairwise distance computation in MEGA software, and DRM analysis in Stanford HIV drug resistance database.

### 2.4. Statistical analysis

Descriptive statistics were used to present median values and interquartile range (IQR) for age and HIV-1 VL. Fisher's exact test was used to assess differences in the distribution of DRMs between Lancet and NHLS samples, with focus on mutations conferring resistance against TLD. A p-value of $\leq$0.05 was considered statistically significant. All statistical tests were performed on the STATA 16.0 software package (StataCorp LP, College Station, TX, USA).

## 3. Results

### 3.1. Demographics

This study tested 59 HIV-1-infected patient samples; 41 from Lancet and 18 from NHLS. The Lancet samples were obtained from patients with a median age of 44 years (IQR: 31 – 51) versus 36 years (IQR: 26 – 45.5) for NHLS samples. The Lancet samples had a median VL of 58,632 copies/ml (IQR: 6010 – 233,905 copies/ml) while NHLS samples had a median VL of 42,500 copies/ml, (IQR: 34,600 – 60,700 copies/ml) (Table 1, Supplementary Table 3).

The in-house multiplex PCR assay detected one or both target fragments in almost all the extracted samples, (Fig. 1). The proportion of samples that had both target fragments detected was 66.1 % (39/59) with similar or varying band intensity on agarose-gel electrophoresis.

### 3.2. Performance of an in-house multiplex PCR assay

The separate evaluation of PR-RT and IN primers showed successful

**Table 1**
Demographics of the study participants and performance of the multiplex PCR assay.

| | NHLS samples (n = 18) | Lancet samples (n = 41) |
|---|---|---|
| Median age (IQR) | 36 years (26 – 45.5) | 44 years (31 – 51) |
| Median VL (IQR) | 42,500 copies/ml (34,600 – 60,700) | 58,632 copies/ml (6010 – 233,905) |
| Country of origin | | |
| • RSA | 18 (100 %) | 28 (68.29 %) |
| • Zimbabwe | none | 1 (2.44 %) |
| • Eswatini | none | 8 (19.51 %) |
| • Botswana | none | 1 (2.44 %) |
| • Kenya | none | 3 (7.32 %) |
| Stratification of participants' samples by VL | | |
| • VL <5000 copies/ml | none | 10 (24.39 %) |
| • VL >5000 copies/ml | 18 (100 %) | 31 (75.61 %) |
| Assay performance per VL category | | |
| IN fragment detection: | | |
| • VL <5000 copies/ml | n/a | 10 (100 %) |
| • VL >5000 copies/ml | 18 (100 %) | 27 (87.10 %) |
| PR-RT fragment detection: | | |
| • VL <5000 copies/ml | n/a | 8 (80 %) |
| • VL >5000 copies/ml | 16 (88.89 %) | 25 (80.65 %) |

NHLS – National Health Laboratory Service; IQR – Interquartile range; RSA – Republic of South Africa; PCR – Polymerase chain reaction; VL – Viral load; PR-RT – protease – reverse transcriptase; IN – integrase; n/a – not applicable. See supplementary Table 1 for more details.

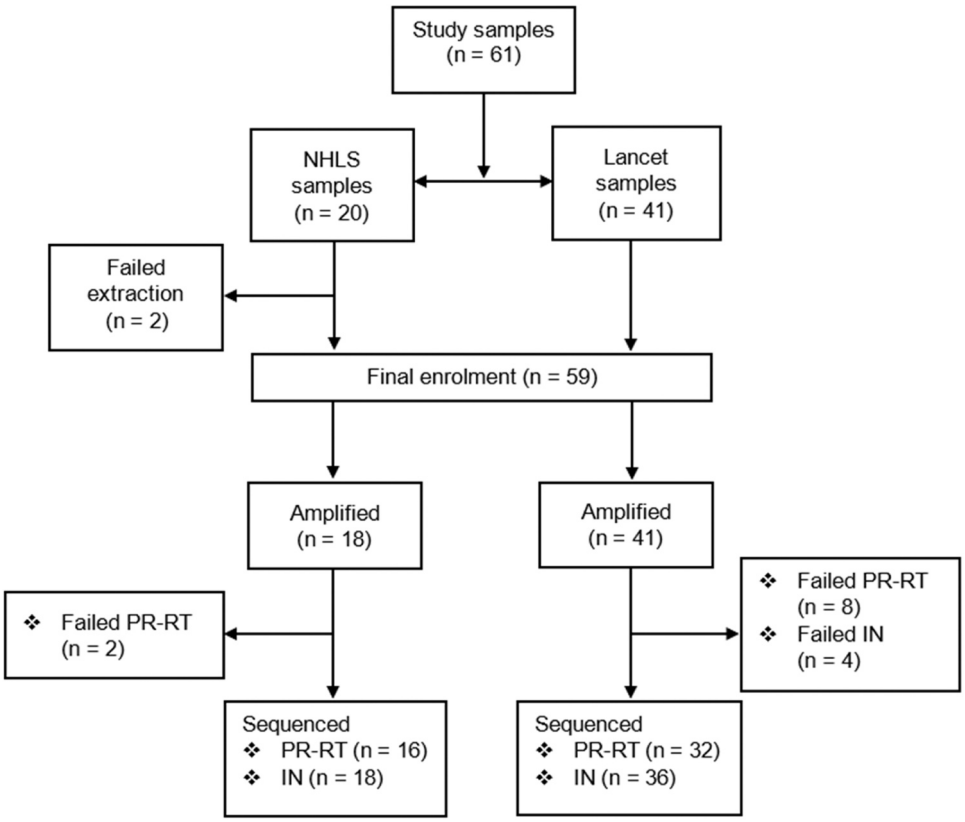


**Fig. 1. :** Flow chart summarising testing of the samples that were used to evaluate the in-house multiplex PCR assay from sample collection to sequencing. NHLS – National Health Laboratory Service; PR-RT – protease-reverse transcriptase; IN – integrase.

amplification of their target fragments (Supplementary Figure 1). These primers were then evaluated in a multiplex PCR protocol which used equal PR-RT and IN primer concentrations in the first and second rounds. The multiplex PCR assay successfully detected the PR-RT and IN targets but demonstrated a bias towards the amplification of the smaller IN fragment. We optimised the multiplex PCR to control for this bias by lowering the IN primer concentration to 0.14 pmol/µl (0.35 µl) while using 0.2 pmol/µl (0.5 µl) of PR-RT primers, and this corrected the bias in most samples (Fig. 2).

Few other protocols which used unequal primer concentrations [e.g. 0.28 pmol/µl (0.7 µl) PR-RT and 0.16 pmol/µl (0.4 µl) IN] in the second-round PCR were also evaluated. We later found that the multiplex PCR protocols which used unequal primer concentrations in the second-round shifted bias towards PR-RT in some samples. We then reverted back to using the equal primer concentrations [0.2 pmol/µl (0.5 µl) for both PR-RT and IN] in the second-round PCR so as to ensure that the IN fragment amplification is not compromised. This led to a more consistent detection of IN in almost all samples (Fig. 3).

In samples in which the multiplex PCR assay was not successful in detecting target fragment(s), a separate second-round PCR was prepared using amplicons from the first-round reaction and single target-specific primers, and this was mostly employed for the PR-RT fragment due to the bias towards the IN fragment in the multiplex PCR assay. This yielded desirable results in most cases (Supplementary figure 2). PR-RT target amplification failure was observed in fourteen samples, nine of which had VL >5000 copies/ml. IN target amplification failure was observed in five samples, all of which had VL >5000 copies/ml (Table 1).

### 3.3. Sensitivity, precision and reproducibility analysis

Four-fold serial dilutions were prepared on two different occasions to assess the sensitivity of the in-house multiplex PCR assay using a sample with a VL of 61,400 copies/ml. The in-house multiplex PCR assay was able to consistently detect target fragment(s) up to 1/64 dilution on both occasions. Therefore, the detection limit of the in-house multiplex assay is 960 copies/ml. However, target fragment(s) were detected in 1/256 dilution on one occasion (Fig. 4). The PCR amplification success was observed in few samples with VL <1000 copies/ml (Supplementary figure 3). Pairwise distance data showed 100 % sequence similarity and ≥99.9 % sequence similarity for sequences used for precision and reproducibility analysis, respectively (Supplementary figure 4). The DRM results were also similar in all samples used for precision and reproducibility analysis (Supplementary Table 4).

### 3.4. Phylogenetic analysis

Phylogenetic analysis showed that the majority of PR-RT and IN sequences (96 % and 94 %, respectively) clustered with HIV-1 subtype C references. Interestingly, in a few samples (2/48, 4 %) it was noticed that PR-RT sequences clustered with non-subtype C reference strains but the corresponding IN sequences clustered with subtype C reference strains (Supplementary figure 5).

### 3.5. ARV drug resistance between in-house vs Lancet sequence data

ARV drug resistance data obtained from sequencing the in-house multiplex PCR amplicons was compared with data from Lancet samples and this analysis was performed in 32 PR-RT sequences and 36 IN sequences (Fig. 1). There was 100 % concordance for the detection of INSTI mutations, but this was lower (90.3 %) for PR-RT mutations due to discordant results in three (9.7 %) samples (sample 5969, 5038 and 2978) (Table 2). Repeat sequencing from PCR amplicons yielded the same results for all these samples.

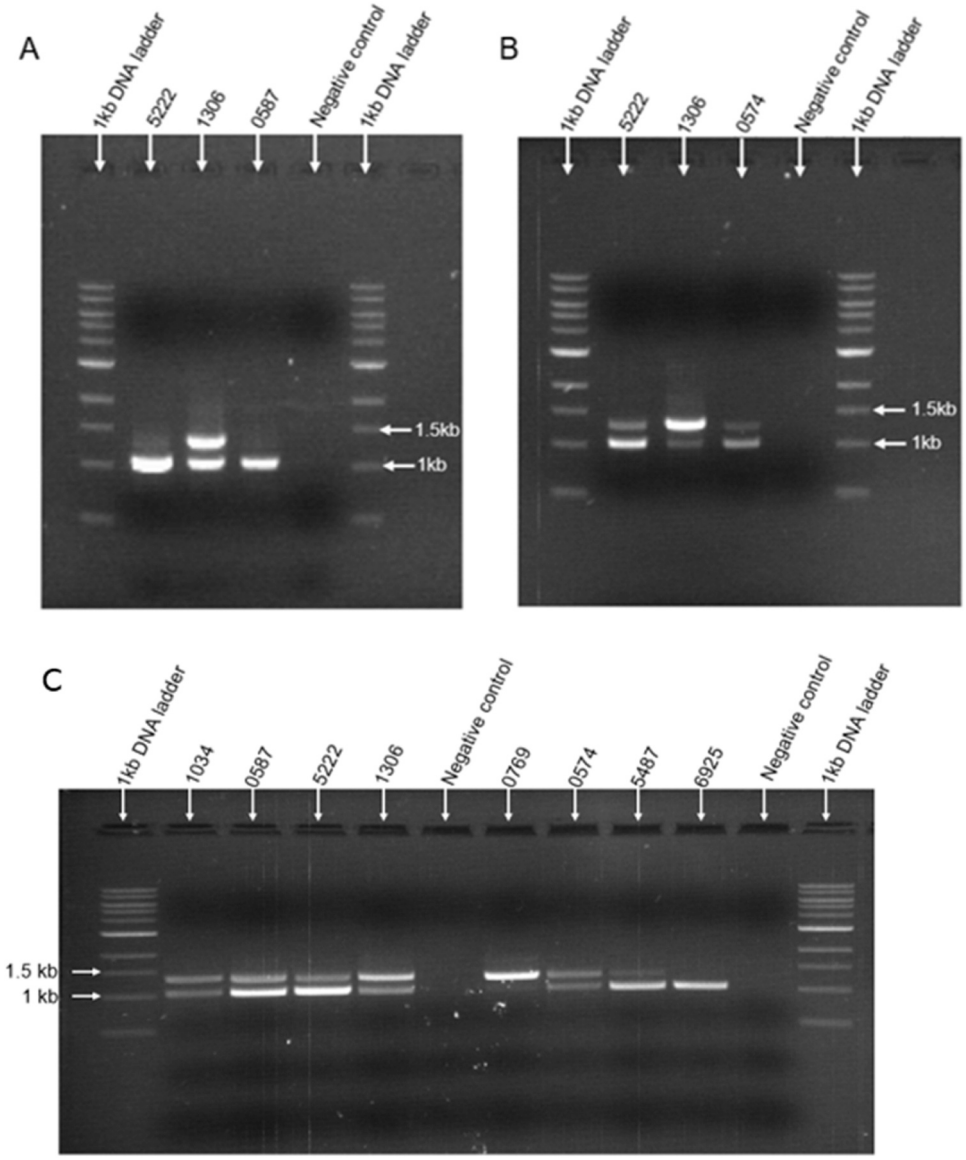


**Fig. 2.** Agarose gel electrophoretogram showing (A.) Multiplex PCR assay results when equal PR-RT and IN primer concentrations [0.2 pmol/μl (0.5 μl) for both PR-RT and IN] were used in the second-round, which demonstrated a bias towards the smaller IN fragment (1 kb). (B.) Multiplex PCR assay results when the second-round IN primer concentration was lowered to 0.14 pmol/μl (0.35 μl) which corrected the bias as reflected by the detection of both fragments. (C.) Amplification of project samples using optimised multiplex PCR protocol, which yielded desired bands in almost all samples except for 6925 that had no PR-RT fragment. Sample 0769 had a fainter IN band. DNA – deoxyribonucleic acid; kb – kilobase. PR-RT – Protease-reverse transcriptase; IN – integrase.

### 3.6. ARV drug resistance data in NHLS samples

For NHLS samples, final sequence analysis included sixteen PR-RT sequences and eighteen IN sequences (Fig. 1). The in-house multiplex PCR sequence data was able to detect DRMs in seven (43.8 %) of the NHLS samples. The most common major mutation detected was an NRTI mutation, M184V, which was observed in 6 samples (37.5 %). Other mutations conferring resistance to NRTI, NNRTI and PI drug classes were observed in fewer samples. There were no INSTI mutations detected (Table 3).

### 3.7. Mutations that confer resistance against TLD

The proportion of some DRMs conferring resistance to the current first-line regimen was generally higher in Lancet sequences compared to NHLS sequences. An M184V mutation was detected in 21/31 (67.7 %) Lancet sequences compared to 6/16 (37.5 %) in NHLS sequences (p = 0.04). K65R was detected in 3/31 (9.7 %) Lancet sequences and in 2/16 (12.5 %) NHLS sequences (p = 1.000). Major INSTI drug resistance mutations (G118R and R263K) were observed in five Lancet sequences and none of these were observed in NHLS sequences (p = 0.157) (Tables 2 and 3).

### 3.8. Cost-estimate analysis

Cost analysis was performed based on the prices of 100 reaction SSIII RT-PCR kit (United States dollar [USD] 652) and SuperFi enzyme (USD154), which are the more expensive PCR reagents. At the time of analysis, 1 USD was equivalent to R19 (South African Rand). The cost per reaction was USD7 and USD2 for SSIII RT-PCR kit and Platinum SuperFi enzyme, respectively. Based on this, the total cost of amplifying 59 samples with a nested PCR that amplifies PR/RT and IN separately (first and second round, total of four reactions per sample) would be USD1888, excluding amplification failures. This cost would be halved

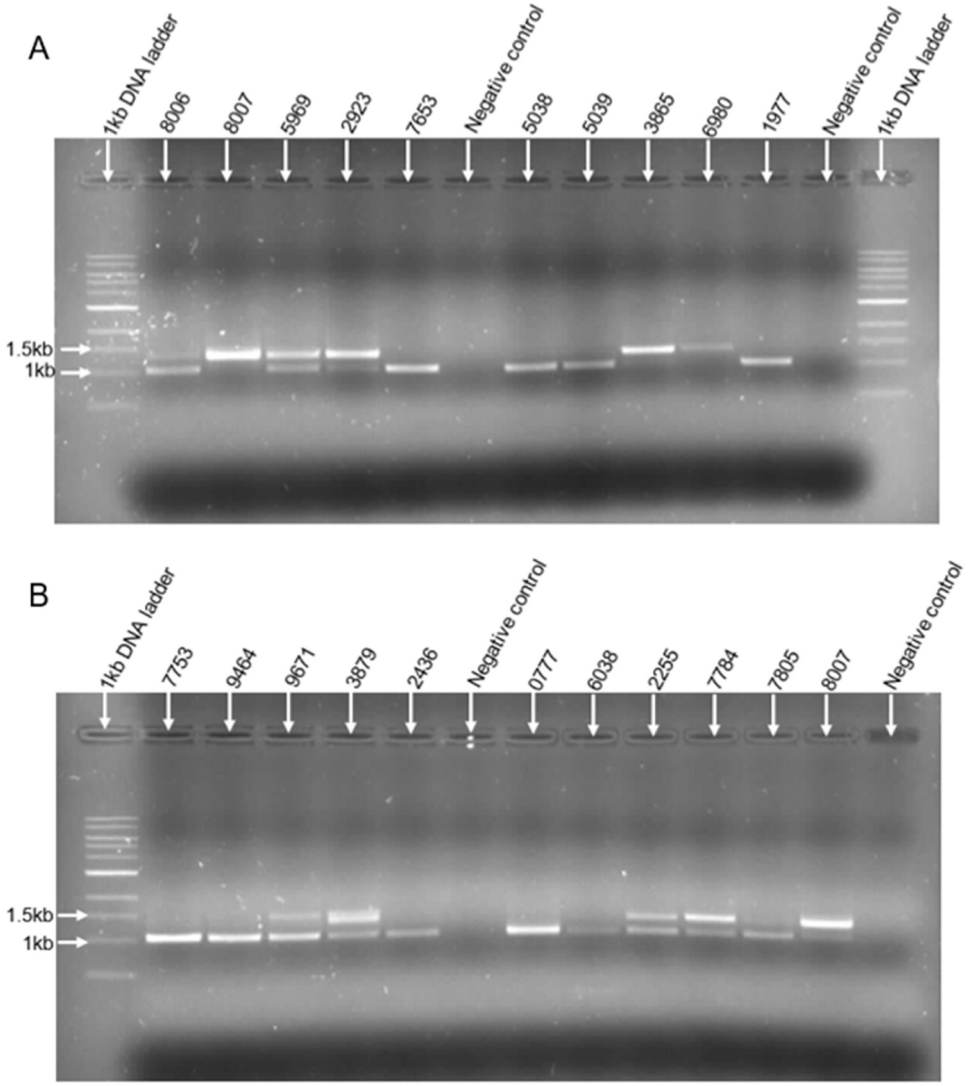


**Fig. 3.** Agarose gel electrophoretogram showing the multiplex PCR results: (A.) Using unequal primer concentrations in the second-round PCR, which shifted bias towards PR-RT fragment. (B.) Using equal PR-RT primer and IN primer concentrations in the second-round, which led to consistent detection of the IN fragment. A separate PR-RT nested PCR assay was prepared for samples in which PR-RT was not detected with the in-house multiplex PCR assay. DNA – deoxyribonucleic acid; kb – kilobase.

(USD944) with the in-house multiplex PCR if there were no amplification failures. However, separate second round PCR reactions were performed in 16 samples that had initially failed amplification, this increased the cost of using the in-house multiplex PCR from USD944 to USD1088. This translates to ~42 % cost saving compared to assays that amplify PR/RT and IN separately. The in-house multiplex PCR used a total of 8 sequencing primers to sequence each sample, which is comparable to what is used in other HIV drug resistance assays (Manyana et al., 2023), as such, no costs would be saved on sequencing.

## 4. Discussion

To our knowledge, this is the first study to develop a conventional multiplex PCR assay that simultaneously detects both PR-RT and IN fragments. The major advantage of the in-house PCR assay is that it significantly reduces the cost of PCR testing. Currently, most PCR assays used for HIV-1 drug resistance testing amplify PR-RT separately from IN, which is more expensive and therefore unsuitable for resource-limited settings (Rhee et al., 2016; Obasa et al., 2020; Rosemary et al., 2018; Delaney et al., 2023). The other advantage of this in-house multiplex PCR assay is that it has a lower detection limit of 960 copies/ml, and thus can reliably detect target fragments in samples with VL >1000 copies/ml, which is a cut-off that is commonly recommended for ARV drug-resistance testing in most guidelines (SADH, 2023; WHO, 2021; Steegen et al., 2023; Medical Care Criteria C, 2020). This assay seems promising in detecting targets in samples with a VL <1000 copies/ml as it detected a target fragment in 1/256 dilution (~240 copies/ml) during sensitivity analysis and was successful in some samples with VL <1000 copies/ml, showing that there is a possibility that its performance in low VL samples can be further optimised. The in-house multiplex PCR assay also demonstrated excellent precision and reproducibility. Most samples that failed amplification had VL >5000 copies/ml (Table 1), showing that this is probably due to primer mismatch which could be improved with primer modification / design.

The in-house multiplex PCR assay demonstrated greater sensitivity (93.2 %) for detection of the IN fragment in study samples. The benefit of this is that INSTI resistance data is much more important in the era where DTG is favoured for HIV-1 treatment as patients still achieve full VL suppression when DTG is used with NRTI drugs from a failing regimen even with documented NRTI drug-resistance mutations (Paton et al., 2022; Keene et al., 2021; Schramm et al., 2022). Other researchers have managed to amplify the entire HIV-1 *pol* gene as a 2.9 kb fragment

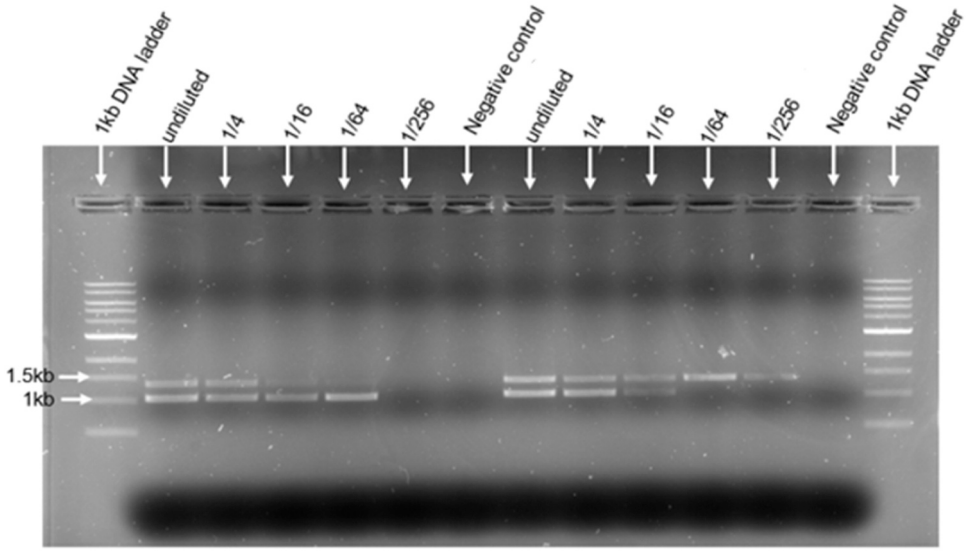


**Fig. 4. : Agarose gel electrophoretogram showing results for the sensitivity testing.** A four-fold serial dilution of sample 5434 was used for sensitivity analysis. This sample had a VL of 61,400 copies/ml and was extracted using a 0.5 ml input volume. A 5 μl volume from the dilution series and the undiluted sample was used as template for the first-round and a 0.5 μl volume from the first round was used as a template in the second-round PCR. The in-house multiplex PCR assay was able to detect target fragment(s) up to 1/64 dilution in the initial sensitivity testing, and up to 1/256 dilution in the repeat testing. A 5ul volume of undiluted sample contained 2558 RNA copies, thus the VL in 1/4, 1/16, 1/64 and 1/256 dilutions were 640, 160, 40 and 10 RNA copies, respectively. Detecting target fragment(s) in these dilutions translates to a VL of 15,340 copies/ml, 3838 copies/ml, 960 copies/ml and 240 copies/ml, respectively. The in-house multiplex PCR assay was able to consistently detect target fragment(s) up to 1/64 dilution, thus the detection limit of the in-house multiplex PCR is estimated to be 960 copies/ml. kb – kilobase; DNA – deoxyribonucleic acid.

in a singleplex PCR, however this assay demonstrated primer dimers, nonspecific amplification and poor performance in low VL samples (Manyana et al., 2023). Previous data from our research team showed that a nested singleplex PCR that amplified the entire HIV-1 *pol* gene (3.2 kb) had a higher detection limit of 2600 copies/ml (Nkone et al., 2022). The in-house multiplex PCR assay has a lower detection limit and did not show any primer dimers or non-specific amplification.

The in-house multiplex PCR protocol with equal PR-RT and IN primer concentrations demonstrated a bias towards the amplification of the IN fragment. Reasons for this bias could include that the IN fragment is a shorter target, differing primer GC content, presence of secondary structures in the genome, or unequal distribution of primers during PCR master mix preparation (Liu et al., 2020; Elnifro et al., 2000).

Phylogenetic analysis has shown that the majority of PR-RT and IN project sequences clustered with HIV-1 subtype C reference strains. This was expected as this is the most prevalent subtype in the southern African region (Bbosa et al., 2019; Wilkinson et al., 2015). A small number of PR-RT sequences clustered with non-subtype C strains, but their corresponding IN sequences clustered with HIV-1 subtype C strains indicating possible recombination as HIV-1 is a highly diverse virus (Adeniyi et al., 2021; Kiwelu et al., 2013). This also highlights that the in-house multiplex PCR is able to detect non-subtype C strains.

There was 100 % concordance for detection of INSTI DRMs between ARV drug-resistance data obtained from Lancet and in-house multiplex PCR assay, however, this was lower in PR-RT (90.3 %) due to discordant results in 3 sequences. These results would not affect the choice of regimen in the absence of major INSTI mutations as DTG-containing regimen has been effective in suppressing VL in patients with NRTI resistance mutations who failed a previous regimen (Paton et al., 2022; Keene et al., 2021; Schramm et al., 2022). The major INSTI resistance mutations were only found in Lancet samples (Table 2) but this was not statistically significant. RAL has been included in the South African private sector ART guidelines since 2013 (NDoH, 2013), and has a low genetic barrier to resistance, thus use of this drug could be a risk factor for the emergence of DTG drug resistance in the private sector (Xiao et al., 2023; Tao et al., 2023; Delaugerre, 2010). DTG was only adopted in the South African public sector in 2019 and this could explain the absence of INSTI mutations in NHLS samples (SADH, 2019; WHO, 2019; Dorward et al., 2023).

Limitations of this study include a small sample size, and that the majority of sequences belonged to HIV-1 subtype C. Thus, the performance of the in-house multiplex PCR assay is not known yet in HIV non-subtype C strains. The in-house multiplex PCR assay failed to amplify target fragment(s) in a minority of samples possibly due to primer mismatches. Discordant drug resistance results were observed in a few samples for the PR-RT sequences. Unfortunately there were insufficient samples for repeat testing. This study evaluated the in-house multiplex PCR assay in randomly selected samples, thus its drug resistance data in both NHLS and Lancet samples cannot be generalised.

## 5. Conclusions

This study demonstrated a satisfactory performance of a conventional multiplex PCR assay for HIV-1 drug resistance testing. This could be a cost-effective method for HIV-1 drug resistance testing especially in resource-constrained settings. The other advantage of this assay is the higher sensitivity of detection of IN fragment, which would guarantee availability of INSTI drug-resistance data in most samples in an era where DTG-containing regimen is preferred for HIV-1 treatment. Further studies are needed for optimisation of this in-house multiplex PCR assay.

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**Table 2**
Comparison between in-house and Lancet laboratory ARV drug resistance results.

| Sample no | Subtype | Lancet resistance results | | | | In-house resistance results | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **PI** | **NRTI** | **NNRTI** | **INSTI** | **PI** | **NRTI** | **NNRTI** | **INSTI** |
| 8006 | C | None | M184V | K103N, P225H | G118R | None | M184V | K103N, P225H | G118R |
| 8007 | C | None | K70Q, L74I, Y115F, M184V | V106M, V179D | None | None | K70Q, L74I, Y115F, M184V | V106M, V179D | None |
| 7753 | C | None | V75M | K101E, K103N, E138K | None | No PR-RT sequence | | | None |
| 9464 | C | None | M184V | None | None | None | M184V | None | None |
| 9671 | C | None | None | Y188L | None | None | None | Y188L | None |
| 5969* | C | M46I, I50L, V82M, L90M | K65R, M184V, K219Q | E138A, V179D | None | M46L, I50L, V82M | K70R, M184V, K219Q | E138A, V179D, K103R | None |
| 3879 | C | None | D67N, K70R, M184V, T215V, K219E | K101E, V108I, Y181C, G190A | None | None | D67N, K70R, M184V, T215V, K219E | K101E, V108I, Y181C, G190A | None |
| 2436 | A | None | K70Q, M184V | None | No IN sequence | None | K70Q, M184V | None | None |
| 2923 | C | None | None | None | None | None | None | None | None |
| 0777 | C | None | K65R, V75M, M184V | K101P, K103N/S, E138Q | None | None | K65R, V75M, M184V | K101P, K103S, E138Q | None |
| 7653 | C | None | None | None | None | No PR-RT sequence | | | None |
| 6038 | C | None | None | K101E, Y181C, G190A | R263K | No PR-RT sequence | | | R263K |
| 5038* | C | None | A62V, V75I, M184V | None | None | None | M184V | None | None |
| 5039 | C | None | M41L, D67S, K70R, M184V, K219E | V106M, Y188L | G118R | None | M41L, D67S, K70R, M184V, K219E | V106M, Y188L | G118R |
| 2255 | C | None | K70R, M184V | None | G118R | None | K70R, M184V | None | G118R |
| 3865 | C | None | None | None | None | None | None | None | None |
| 7784 | D | None | None | K103N, E138G | No IN sequence | None | None | K103N, E138G | None |
| 6980 | C | M46I | K65R, M184V | K103N, E138Q, V179L, P225H | No IN sequence | None | M184V | K103N, E138G, V179L, P225H | None |
| 7805 | C | None | None | K103N | No IN sequence | No PR-RT sequence | | | None |
| 1977 | A | None | M41L, K65R, S68G, V75M, M184V | K103S, V179T, G190S | No IN sequence | No PR-RT sequence | | | G118R |
| 5267 | C | None | None | None | None | None | None | None | None |
| 1823 | C | G48V, I54V, V82T, I84V | K70EQ, M184V | K103N | None | G48V, I54V, V82T, I84V | K70E, M184V | K103N | No IN sequence |
| 2977 | C | M46I | M41L, D67N, K70S, V75M, M184V, T215Y | K103N, K238N | None | M46I | M41L, D67N, K70G, V75M, M184V, T215Y | K103N | None |
| 2354 | C | None | None | K103N, P225H | None | NS | | | |
| 0359 | C | None | K70PT, M184V | A98G, P225H | None | None | K70T, M184V | A98G, P225H | None |
| 4345 | C | None | M184V | K103N, P225H, Y318F | None | NS | | | |
| 4316 | C | None | D67G, S68G, K70R, L74I, M184V, T215I, K219E | L100I, K103N | None | None | D67G, S68G, K70R, L74I, M184V, T215I, K219E | L100I, K103N | None |
| 4310 | C | None | M184V | None | None | None | M184V | None | No IN sequence |
| 1721 | C | None | M184V | K103S, V106M, E138A | None | None | M184V | K103S, V106M, E138A | None |
| 8714 | C | None | None | K103N | None | None | None | K103N | None |
| 46980 | C | None | None | K103N | None | None | None | K130N | No IN sequence |
| 5972 | C | None | None | K103N | None | NS | | | None |
| 5028 | C | None | K70R, M184V, K219Q | K103N, P225H | None | None | K70R, M184V, K219Q | K103N, P225H | None |
| 9286 | C | None | None | V106I, Y188L | None | None | None | V106I, Y188L | None |
| 5428 | C | None | A62AV, K65KR, L74I, M184V | L100LI, K103N, V106VA, P225H | No IN sequence | None | A62V, K65R, M184V | L100I, K103N, V106A, P225H | None |
| 3260 | C | None | None | Y181C | None | None | None | Y181C | None |
| 0958 | C | G48V, I54V | K65R, M184V, K219Q | V106I, Y181C | No IN sequence | G48V, I54V | K65R, M184V, K219Q | V106I, Y181C | None |
| 2978* | C | None | None | V106VM, G190A | None | None | None | G190A | None |
| 2229 | C | M46I, I50V, I54V | K70R, M184V, K219Q | A98G, K103N, P225H | No IN sequence | M46I, I50V, I54V | K70R, K219Q | A98G, K103N, V179D, P225H | None |
| 3246 | C | None | M184V | None | No IN sequence | None | M184V | None | None |
| 5968 | C | None | M184V | V106M, G190A, F227L | No IN sequence | NS | | | None |

* - Samples with discordances between Lancet laboratory and in-house multiplex PCR resistance data.; NS - No sequence due to failed amplification / sequencing; PI – Protease inhibitor; NRTI- Nucleoside reverse transcriptase inhibitor; NNRTI – Non-nucleoside reverse transcriptase inhibitor; INSTI – integrase strand transfer inhibitor.

**Table 3**
Drug resistance mutations detected in NHLS samples using the in-house multiplex PCR.

| Sample no | Viral load (copies/ml) | Drug resistance mutations | | | |
|---|---|---|---|---|---|
| | | PI | NRTI | NNRTI | INSTI |
| 0587 | 32200 | M46L, I54V, V82A | M184V, T215Y | None | None |
| 1034 | 42000 | None | K70E, Y115F, M184V, K219KR | K103N, E138G, H221HY, P225H, K238N | None |
| 5222 | 75900 | None | None | K103N | None |
| 1306 | 74600 | None | None | None | None |
| 0769 | 45300 | None | None | None | None |
| 0574 | 49900 | None | None | None | None |
| 5487 | 60400 | I50V, I54V, V82A | M184V | K103S, G190A | None |
| 6925 | 40700 | NS | NS | NS | None |
| 6779 | 44000 | N/A | None | None | None |
| 6623 | 38900 | None | K65KR, S68R, M184V, K219E | K103N, Y188C, M230L | None |
| 5405 | 43000 | None | None | None | None |
| 7168 | 60700 | None | M184V | V179D | None |
| 5068 | 31300 | NS | NS | NS | None |
| 5369 | 28000 | None | K65KR, S68R, V75VM, M184V | V106M, G190GA, F227FL | None |
| 5434 | 61400 | None | None | None | None |
| 7859 | 33700 | None | None | None | None |
| 9262 | 34600 | None | None | None | None |
| 0204 | 36700 | L90M | None | V106M, Y188YC | None |

N/A – Not analysed due to poor quality PR sequence; NS – No sequence due to failed amplification; PI – Protease inhibitor; NRTI- Nucleoside reverse transcriptase inhibitor; NNRTI – Non-nucleoside reverse transcriptase inhibitor; INSTI – integrase strand transfer inhibitor.

## CRediT authorship contribution statement

**Caroline T. Tiemessen:** Writing – review & editing, Supervision, Formal analysis. **Keitumetse Moeng:** Writing – review & editing, Formal analysis. **Shayne Loubser:** Writing – review & editing, Formal analysis. **Simnikiwe H. Mayaphi:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Raquel Viana:** Writing – review & editing, Formal analysis. **Allison Glass:** Writing – review & editing, Formal analysis. **Paballo Nkone:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Tumelo L. Fortuin:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

All data generated or analysed during this study are included in this manuscript.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2024.115034.

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