

Next Generation Sequencing Based In-house HIV Genotyping Method: Validation Report

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Methodology

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Abstract

Background

HIV genotyping has had a significant impact on care and treatment of HIV/AIDS. At clinical level, the test guides physicians on the choice of treatment regimens. At surveillance level, it informs policy on consolidated treatment guidelines and microbial resistance control strategies. Until recently, the conventional test has utilized Sanger sequencing (SS) method. Unlike Next Generation Sequencing (NGS), SS is limited by low data throughput and the inability of detecting low abundant drug resistant variants. NGS has the capacity to improve sensitivity and quantitatively identify low-abundance variants; in addition, it has the potential to improve efficiency as well as lowering costs when samples are batched. Despite the NGS benefits, its utilization in clinical drug resistance profiling is faced with mixed reactions. These are largely based on lack of a consensus regarding the quality control strategy. Nonetheless, transitional views suggest validating the method against the gold-standard SS. Therefore, we present a validation report of an NGS-based in-house HIV genotyping method against SS method in Uganda.

Results

Since there were no established proficiency test panels for NGS-based HIV genotyping, fifteen (15) clinical plasma samples for routine care were utilized. The use of clinical samples allowed for accuracy and precision studies. The workflow involved four (4) main steps; viral RNA extraction, targeted amplicon generation, amplicon sequencing and data analysis. Accuracy of 98% with an average percentage error of 3% was reported for the NGS based assay against the SS platform demonstrating similar performance. The coefficient of variation (CV) findings for both the inter-run and inter-personnel precision showed no variability ($CV \leq 0\%$) at the relative abundance of $\geq 20\%$. For both inter-run and inter-personnel, variation that affected the precision was observed at 1% frequency. Overall, for all the frequencies, CV registered a small range of (0-2%).

Conclusion

The NGS-based in-house HIV genotyping method fulfilled the minimum requirements that support its utilization for drug resistance profiling in a clinical setting of a low-income country. For more inclusive quality control studies, well characterized wet panels need to be established.

Background

Globally, antiretroviral therapy campaigns have led to a tremendous reduction in morbidity and mortality (1). Despite this, the risk of virologic failure increases with the emergence and potential transmission of drug resistant variants which threaten the third 90 of the UNAIDS 90-90-90 goals to control the HIV epidemic worldwide (2) (3) (4). Fortunately, interventions such as HIV genotyping that monitor HIV-1 drug resistance (HIVDR) and surveillance of transmitted drug resistance serve a critical role in the fight against HIV/AIDS (5) (6) (7).

HIV Genotyping assays are diverse given the dynamic nature of the technique and driven by a vast range of technologies (8). Currently, commercial assays such as ViroSeq are available in developed countries. However, because of the cost and varying sensitivity across HIV-1 subtypes, these are hardly utilized in resource limited settings such as Uganda (9) (10). Instead, most laboratories develop in-house assays that are affordable and designed to genotype HIV-1 subtypes and circulating recombinant forms (CRFs) that are predominant within their localities.

Conventional HIV genotyping using Sanger sequencing techniques serves as the mainstay for clinical HIVDR testing. Although Sanger sequencing has been commonly applied as the “gold standard” for a while, there are some intrinsic limitations with this technology. As opposed to Next Generation Sequencing, the technique has low data throughput and limited capacity to detect variants below 20% intra-host frequency of the quasi-species (11) (12). Literature suggests that low-abundance HIVDR variants could have a relevant clinical impact and that their detection could benefit treatment management (13) (14) (15). However, a clinically significant threshold is yet to be defined amidst the promising benefits of the new NGS technologies.

Although NGS chemistries differ, all platforms are characterized by high-output, clonal, and parallel sequencing (16). These outperform conventional Sanger sequencing in scalability, sensitivity, and quantitative detection of minority resistance variants (17). The sensitive methodology can accurately profile the protease, reverse transcriptase, integrase, and maturation inhibitors, as well as HIV-1 coreceptor tropism in a single run. Despite this, NGS-based assays have been primarily limited to research settings and are rarely used in clinical settings especially in the low- and middle-income countries. This can be attributed to the assay not being standardized, which is necessary for accreditation by regulatory agencies and lack of an appropriate validation and performance assessment platform for NGS-based HIV genotyping.

Therefore, in reference to the principles of molecular assay validations and benchmark to the available guiding documents (18), here we present a validation report for the NGS-based assay. The validation report was used to assess the HIVDR assay of the Uganda National Health Laboratories Services (UNHLS) which is the national reference laboratory for specialized tests. The report was also used as a benchmark for the NGS assay for Joint Clinical Research Centre (JCRC) which is a WHO-accredited HIVDR laboratory and part of the HIVDR surveillance network.

Results

Accuracy: Accuracy was demonstrated by sequencing ten (10) samples at frequencies above 20% which is the threshold for clinical interpretation. Accuracy of 98% with an average percentage error of 3% was reported for the NGS assay against the Sanger sequencing platform demonstrating similar performance, Table 2. Considering the linear range of mutation detection, no significant difference ($r = 0.99$, $p = 2.5$ Pearson coefficient correlation, Fig. 1) was observed between NGS platform and the gold standard.

Clinical interpretation of drug resistance mutation profile was based on frequencies 20% and above. As expected, the linear range of mutation detection showed a significant correlation when the data indicated a significant difference ($r = 0.7$, $p < 0.05$ Pearson coefficient correlation, Fig. 1) between the two platforms < 20% frequencies. This is attributed to the sequencing depth capacity of the NGS platform as opposed to sanger sequencing.

Precision: Repeatability (inter-run) was demonstrated by processing and sequencing five (5) samples five (5) times using the same conditions in a period of one (1) week. Reproducibility (inter-personnel) was demonstrated by processing and sequencing five (5) samples by five (5) laboratory technologists in a period of one (1) week. The NGS assay passed the precision demonstrations at frequencies of above 20%, discrepancies occurred at very low frequencies of about 1%. As expected, the NGS assay detected similar mutation profiles at 20% frequency which is the threshold for clinical interpretation of drug resistance mutation profiles, Fig. 2. Despite the difference at a low cut off of 1%, the NGS assay detected comparable similar mutation profiles at frequencies lower than 20%. The difference can be attributed to; inter-personnel and experimental errors i.e., pipetting errors, Fig. 2.

The coefficient of variation (CV) was used to determine the inter-run and inter-personnel precision, Table 3. The findings showed no variability ($CV \leq 0\%$) at the relative abundance of 20% for both the inter-run and inter-personnel precision. For both inter-run and inter-personnel, variation that affected the precision was observed at 1% frequency. Overall, for all the frequencies, CV registered a small range of (0%-2%).

Precision demonstrations were further interrogated for the most prevalent mutations that is, M184V and K103N. Figure 3 shows the frequencies of M184V across the precision trials for the five (5) samples. M184V is one of the major and most common mutation among the NRTI class of drugs. M184V causes high-level *in vitro* and *in vivo* resistance to lamivudine (3TC) and emtricitabine (FTC) and low-level resistance to Didanosine and Abacavir (ABC). As expected, frequencies > 20% were relatively uniform across all the runs for every sample. However, at frequencies < 2%, M184V was not visible for some runs. Since the frequencies are too low, the difference can be attributed to; inter-personnel and experimental errors i.e., pipetting errors. Figure 3 also shows the frequencies of K103N across the precision runs for four (4) samples. K103N is one of the major and most common mutation among the NNRTI class of drugs. K103N is a non-polymorphic mutation that causes high-level reductions in Nevirapine and Efavirenz susceptibility. As expected, the frequencies > 20% were relatively uniform across all the runs for every sample. The slight variations are negligible and could be attributed to experimental errors. The fifth sample had no K103N mutation in all the demonstrations.

L74I was the only mutation observed for the Integrase Strand Transfer Inhibitors (INSTI). Figure 4 shows the frequencies across the reproducibility and repeatability runs. L74I is a polymorphic accessory mutation commonly selected by each of the INSTI. Although, a slight difference of ~ 1% was registered, the assay performed as expected. The difference can be attributed to inter-run and inter-personnel bias.

Overall, the average coverage (sequencing depth) per nucleotide position for precision demonstrations was 2795 as also illustrated in Fig. 5.

Discussion

HIV genotyping is increasingly picking pace in Uganda despite resource limitations. This highly specialized molecular test has been a preserve for research purposes for decades which is attributed to operational costs. Mobilization of resources for public health comes at a time when HIV genotyping is advancing technologically. The conventional technology that is currently utilized in the country is based on Sanger sequencing method. The method is faced with limitation such as low data output and inability to detect mutations of the viral minority populations. Despite this, SS still serves as a gold standard primarily because the NGS technologies lack a standardized validation strategy and a consensus is yet to be reached on the relevance of the rich data output.

Nevertheless, UNHLS acquired an NGS platform for use in clinical diagnostics. Therefore, in this report we present a validation report of the in-house assay based on NGS technologies. The assay was validated on two (2) criteria; accuracy and precision. Accuracy of 98% was reported for the NGS assay against the SS assay. The calculations were set at 20% as the mutation frequency threshold given that SS assays can arguably detect mutations in approximately 15% of the viral population variants. Qualitatively all mutations reported on SS were also reported on NGS platform, Table 2. This was expected as NGS is documented to have a higher sequencing depth as compared to SS. The assay can massively generate data without compromising on quality. A significant difference was observed when the threshold was set below 20%, the findings factualize the literature that the NGS platforms can detect mutations occurring at low frequencies as opposed to the SS platforms (17). Although the NGS platform performed as expected against SS, this approach oversimplifies the complexity and richness of NGS HIVDR data that can report on a highly diverse population of HIV. Therefore, well validated External Quality Assurance (EQA) strategies that check the NGS platform remain to be established.

Coefficient of variation was used to study precision. The report documented no variation ($CV \leq 0\%$) at relative abundance of 20%. However, slight variations that affected precision were noted at relative abundances below 20%, but more prominent at 1%. This could be attributed to the sequence error rate of $\sim 1\%$ and the increased likelihood of cross-contamination and sampling/PCR biases (19). Although, minority mutations at frequencies $< 0.5\%$ could result in treatment failure (20), the clinical relevance of variants below 5% is still a question of debate (21). However, if the variants below 5% are proven relevant, the validation process and quality assurance strategies could have to leverage on the use of unique molecular identifiers (UMIs) to determine the accuracy of NGS assays at such lower frequencies (22).

We acknowledge the limitation of using clinical samples as opposed to well characterized wet panels. Because of this, Sensitivity, Specificity and the Lower limit of Detection (LOD) were not studied. Sensitivity determines the probability of the assay to detect mutations when present. Specificity determines the probability of the assay not to detect mutations when absent. LOD determines the lowest

viral load at which the assay can still be able to detect all the mutations present. The true picture of the above-mentioned parameters could not be achieved with clinical samples consisting of viral RNA of variable quality, quantity and variant diversity. However, even for accuracy and precision, a possibility for bias could have been introduced due to the error-prone reverse transcription PCR step and sequencing error (23).

Conclusion

The NGS assay presents opportunities to revolutionize the field of HIV Genotyping. However, these are under exploited partly because, their relevance has not been well studied in the context of clinical management. This has left such a powerful tool to be oversimplified when compared with inferior assays such as SS. In this report we validated the NGS assay against the SS assay. The NGS assay proved fit for HIVDR testing in a clinical setting of a low resource country. However, with the advance in research, a well elaborate quality control strategy needs to be established.

Methods

In the absence of established proficiency test panels for NGS-based HIVDR testing, fifteen (15) clinical plasma samples for routine care were utilized as shown in Table1. These were collected for routine HIV-1 drug resistance testing as standard of care for HIV-infected individuals failing treatment. Briefly in Fig. 6, Viral RNA was extracted using Qiagen QIAmp Viral RNA kit (24). RT-PCR was done using Superscript III One-Step system with Platinum Taq (25). Amplification of the target genes in the pol region was done using two (2) pairs of primers; 1. nested primers covering the protease gene and reverse transcriptase spanning between positions 2155 and 3323; (forward, ACAGCCCCACCAGCAGAG), (reverse, CTGTATATCATTGACAGTCCAGCT). 2. nested primers covering the integrase gene spanning between positions 4022 and 5258; (forward, AGAAGTAAACATAGTAACAGACTCACA) (reverse, TGCAGACCCCAATATGTTCTA) (26). The primer set allows for robustness of the assay with the ability to genotype all circulating HIV-1 subtypes in the country, Table 1. NGS library preparation was done using the Nextera XT DNA library preparation kit (27) and pooled libraries were run using the Miseq V2 reagent kit on the Miseq system (28) at UNHLS. Sanger sequencing was done using BigDye Terminator v3.1 cycle sequencing kit (29) on the ABI genetic analyzer 3730xl (30) at JCRC.

Data Analysis

Data generated was in two (2) formats; FASTA and FASTQ depending on the sequencing platform. FASTA formats are generated from the sanger sequencing platforms. These are text-based formats for representing nucleotide sequences by single-letter codes. Analysis of the FASTA files was done using the web-based RECall as recommended by World Health Organization (WHO) for laboratories in the HIVDR network (31). RECall is an automated base calling software (32). This enabled us to do quality checks on; raw chromatogram quality, single-stranded coverage, sequence length, stop codon, excessive mixtures, bad insertions, ambiguous nucleotide/amino acids, APOBEC mutations, atypical mutations and genetic

distance. Among the outputs of the RECall analysis included; a consensus sequence, an excel file with the mutation list, csv file with the susceptibility scores and pdf files including an image of a neighbor-joining tree.

FASTQ formats were generated from the NGS platforms. These are text-based formats for nucleotide sequences and its corresponding quality scores. Analysis of the FASTQ files was done using an online based pipeline, Hydra (33). Among the outputs from Hydra included; a consensus sequence and an Amino Acid Variant Format (AAVF) file. The AAVF file report provides a compact summary of the amino acid variation obtained by conceptual translation of the NGS read pileup across the examined region of the HIV genome (34). The AAVF files for the samples were then uploaded in the Stanford University HIV Drug Resistance Database (35) for drug resistance profiling.

Criteria for assessment of the HIVDR NGS based home-brewed assay

Accuracy is defined as the closeness of the measurements to the gold standard. Accuracy was demonstrated by sequencing ten (10) samples on both platforms. Precision is defined as how close the agreement of the outcomes is between repeated measurements. This was categorized into: Repeatability -the variation arising when all efforts are made to keep conditions constant by using the same instrument and operator, and repeating the measurements during a short time period. Reproducibility -the variation arising using the same measurement process among different instruments or operators over longer time periods. Repeatability was demonstrated by processing and sequencing five (5) samples five (5) times using the same conditions in a period of one (1) week. Reproducibility was demonstrated by processing and sequencing five (5) samples by five (5) laboratory personnel in a period of one (1) week designated by the initials AA, FK, IN, NE, PA.

List Of Abbreviations

APOBEC Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like gene

AAVF Amino Acid Variant Format

NGS Next Generation Sequencing

SS Sanger Sequencing

UNHLS Uganda National Health Laboratory Services

JCRC Joint Clinical Research Center

PCR Polymerase Chain Reaction

NNRTI Non-nucleoside reverse transcriptase Inhibitors

EQA External Quality Assessment

INSTI Integrase Strand Transfer Inhibitors

DRM Drug Resistance Mutations

HIVDR HIV drug resistance

Declarations

Ethics approval and consent to participate

This study data was obtained following permission to develop an HIV genotyping protocol. The need for approval was waived by the research ethics committee of Uganda National Health Laboratory Services, Kampala Uganda.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files]

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

This work was carried out in collaboration between all authors. Alisen Ayitewala (AA) designed the protocols and drafted the methodology. Charles Kiyaga (CK) and Isaac Ssewanyana (IS) performed the literature search and data review. AA wrote the first draft of the manuscript and managed manuscript revisions. All authors read and approved the final manuscript.

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Tables

Table 1

Experimental sample Characteristics

Sample ID	HIV-1 subtypes	Demonstrations	Sequenced Genes
DR-968-19	D	Accuracy	RT, PR
DR-969-19	D	Accuracy	RT, PR
DR-970-19	A	Accuracy	RT, PR
DR-971-19	D	Accuracy	RT, PR
DR-972-19	A	Accuracy	RT, PR
DR-975-19	A	Accuracy	RT, PR
DR-976-19	B	Accuracy	RT, PR
DR-979-19	B	Accuracy	RT, PR
DR-980-19	D	Accuracy	RT, PR
DR-981-19	C	Accuracy	RT, PR
DR-134-20	A	Precision	RT, PR, INT
DR-138-20	C	Precision	RT, PR, INT
DR-139-20	D	Precision	RT, PR, INT
DR-143-20	D	Precision	RT, PR, INT
DR-289-20	A	Precision	RT, PR, INT

Table 2

Drug resistance mutation profiles for accuracy of NGS against Sanger Sequencing

Sample ID	Sequencing platform	RT region		PR region	
		NRTI mutations	NNRTI mutations	Major mutations	Accessory
DR-968-19	Sanger Sequencing	K70R, M184V, K219Q	K103N, H221Y, M230L, L234I	None	None
	NGS	K70R, M184V, K219Q, D67G	K103N, H221Y, M230L, L234I	None	None
DR-969-19	Sanger Sequencing	None	K103KNRS	None	None
	NGS	None	K103KNRS	None	None
DR-970-19	Sanger Sequencing	M41L, D67N, M184V, T215F	A98G, G190A	N88S	V32A, L33F
	NGS	M41L, D67N, M184V, T215FIS	A98G, G190A	N88S	V32A, L33F
DR-971-19	Sanger Sequencing	M41L, K65R, M184V, K219N	L100I, K103N	None	None
	NGS	M41L, K65R, M184V, K219N, T215FIS	L100I, K103N, G190A, A98G	N88S	V32A, L33F
DR-972-19	Sanger Sequencing	D67N	K103N, K238T, Y188N	None	None
	NGS	D67GNS	K103N, K238T	None	None
DR-975-19	Sanger Sequencing	M41L, M184V, L210W, T215F	A98G	M46I, I50V, I54V, V82A	L10F, L33F
	NGS	M41L, M184V, L210W, T215F	A98G, V108I	M46I, I50V, I54V, V82A	L10F, L33F
DR-976-19	Sanger Sequencing	M184V	K101E, G190A, E138A	None	None
	NGS	K70R, M184V, K219Q	K101E, G190A, K103N, H221Y, M230L, L234I	None	None
DR-979-19	Sanger Sequencing	T69DN, K70R, M184V	G190S	None	None
	NGS	T69DN, K70R, M184V	G190S	None	G48R
DR-980-19	Sanger Sequencing	M184V, T215Y	K101E, E138A, G190A	None	None

	NGS	M184V, T215NSY	K101E, E138A, G190A, H221Y, P225H	M46I, L76V, I84V	Q58E
DR-981-19	Sanger Sequencing	E44D	None	None	None
	NGS	E44D, K65E, M184IV	A98G, K103N, P225H	I54V, V82A	None

Table 3

Coefficient of Variation for inter-run and inter-personnel precision

Coefficient of Variation, CV %					
DRM Relative abundance (% Frequency)					
Precision	1	2	5	10	20
Inter-Run	2%	1%	1%	2%	0%
Inter-Personnel	2%	2%	1%	1%	0%

Figures

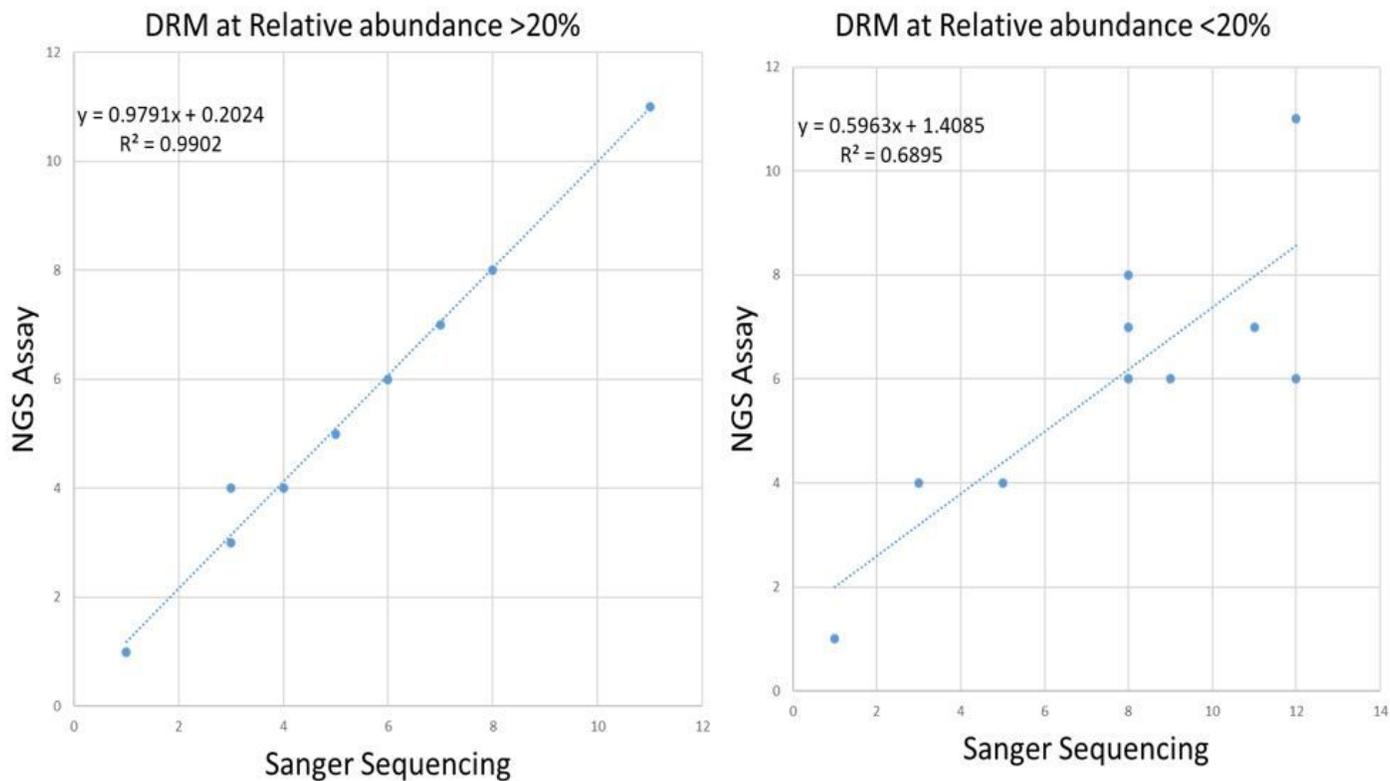


Figure 1

Accuracy demonstrations at relative abundances >20% and <20% Shows correlation between the NGS platform and the gold standard for linear range of mutation detection

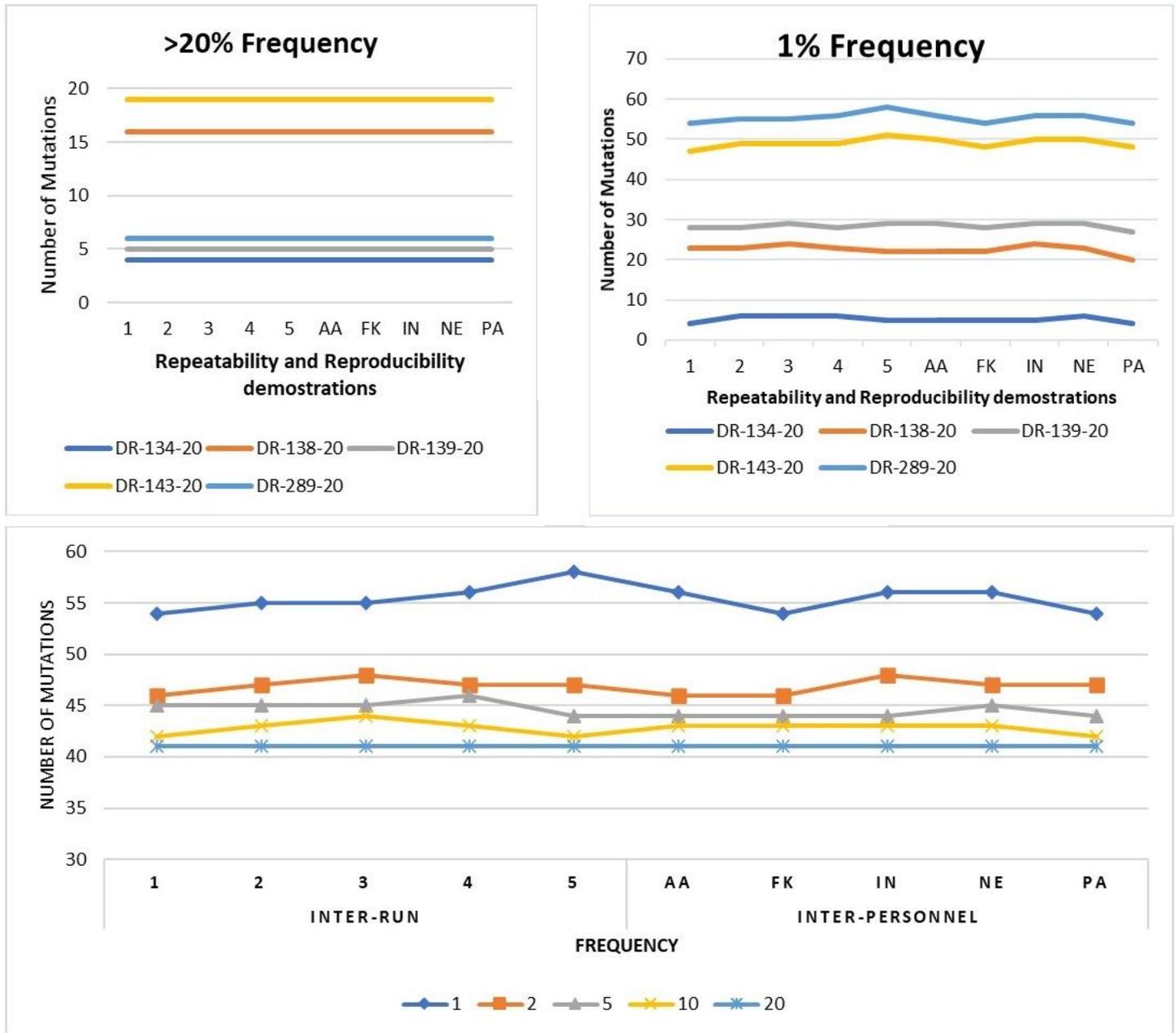


Figure 2

Precision demonstrations for repeatability and reproducibility Shows the detected mutations at different relative abundances for the inter-run and inter-personnel precision trials

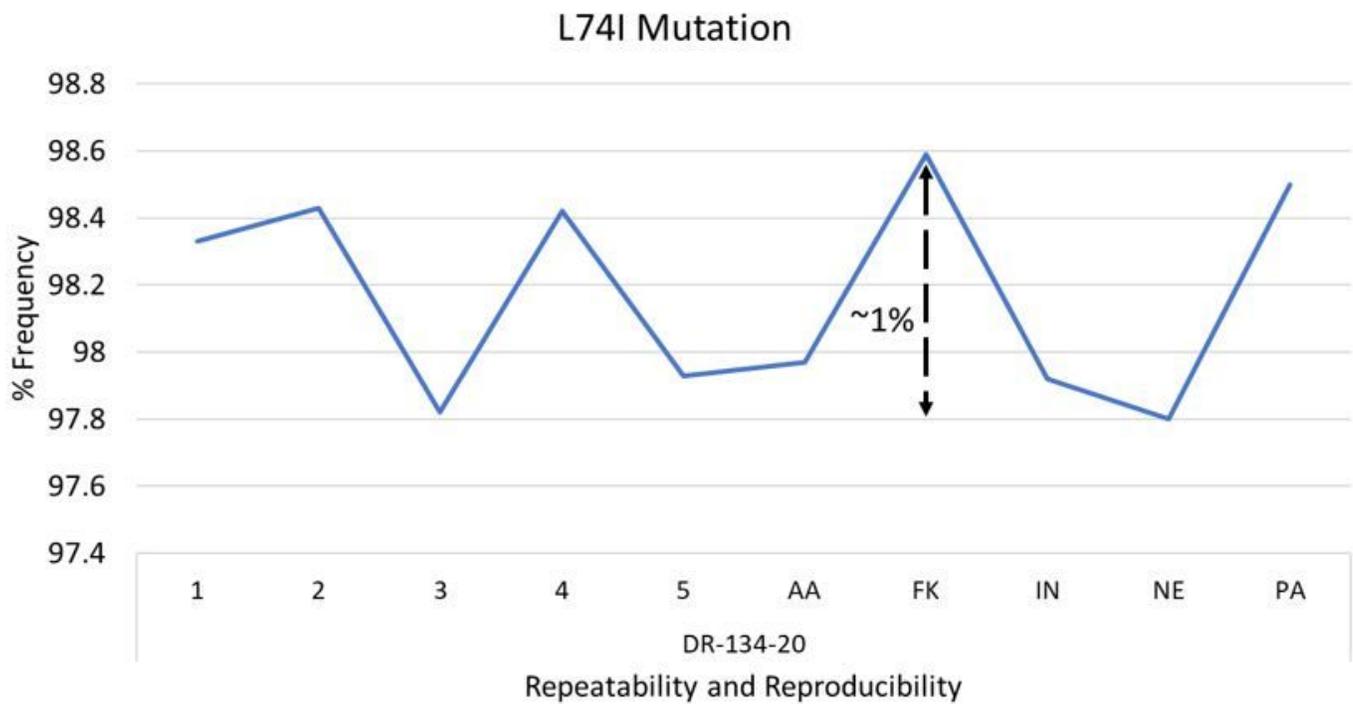


Figure 4

Precision demonstration for L74I mutation Shows the percentage difference of frequencies across precision trials for the integrase inhibitor mutation L74I

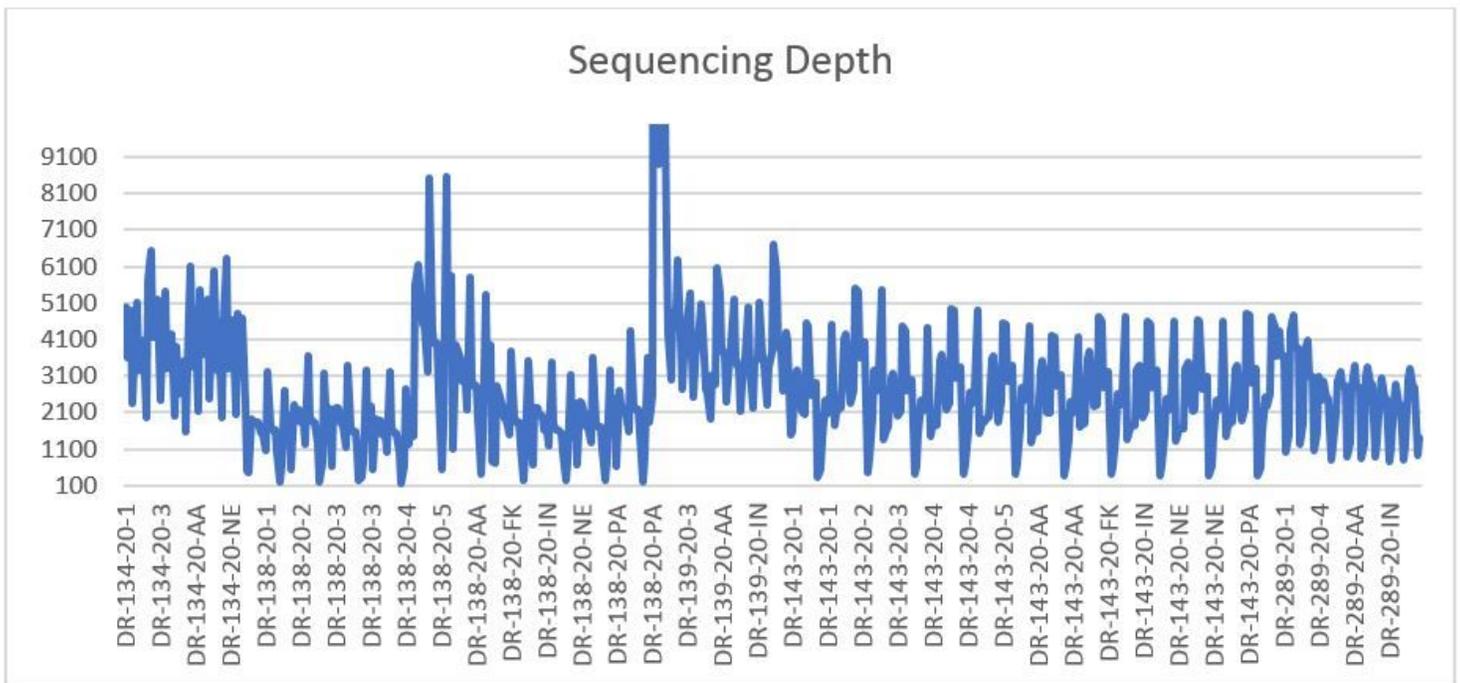


Figure 5

Sequencing depth variations across precision demonstrations Shows an overview of the sequencing coverage for the precision trials

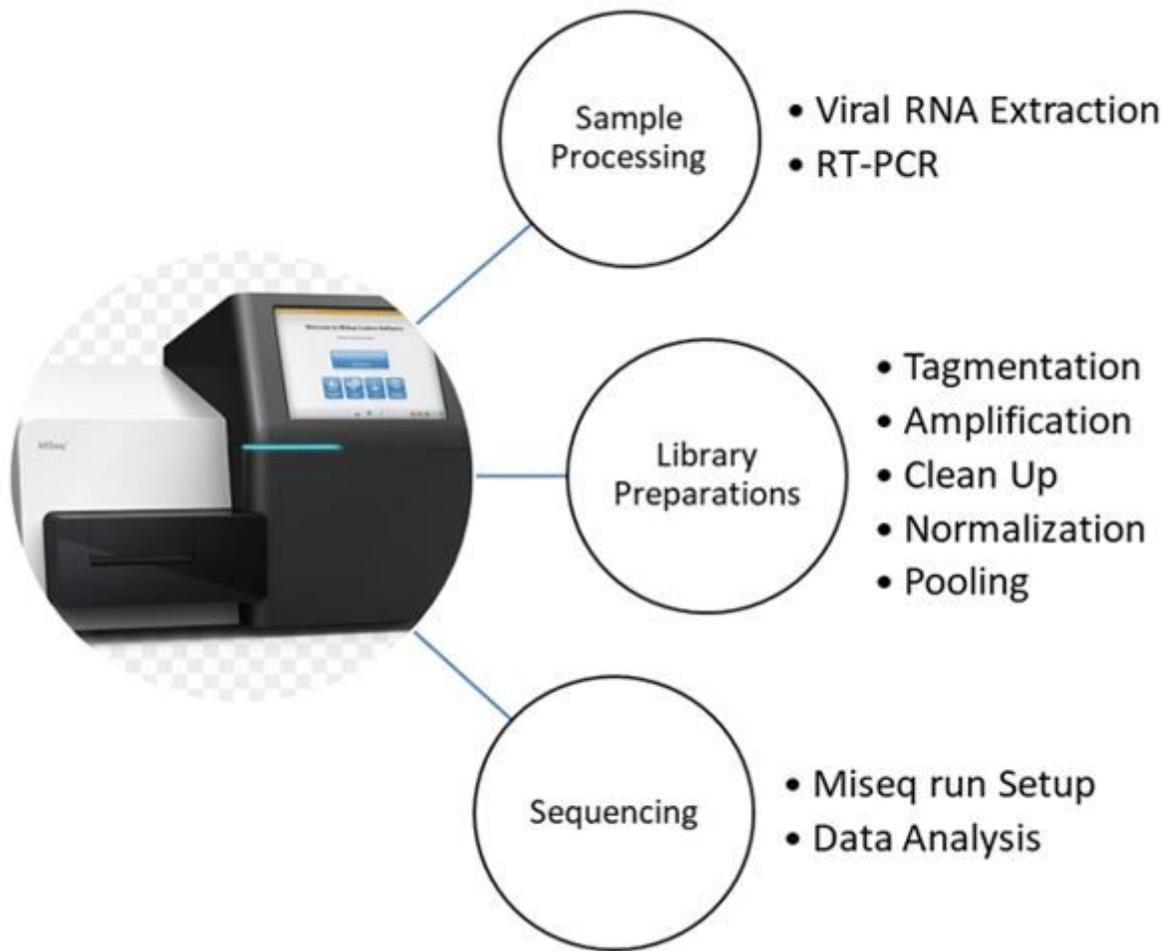


Figure 6

Pictorial view of HIV genotyping processes Shows the experimental steps for NGS platform from viral RNA extraction to analysis of data

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [csvfile.xlsx](#)