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Comparison of an *In Vitro* Diagnostic Next-Generation Sequencing Assay with Sanger Sequencing for HIV-1 Genotypic Resistance Testing

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ABSTRACT The ability of next-generation sequencing (NGS) technologies to detect low frequency HIV-1 drug resistance mutations (DRMs) not detected by dideoxynucleotide Sanger sequencing has potential advantages for improved patient outcomes. We compared the performance of an *in vitro* diagnostic (IVD) NGS assay, the Sentosa SQ HIV genotyping assay for HIV-1 genotypic resistance testing, with Sanger sequencing on 138 protease/reverse transcriptase (RT) and 39 integrase sequences. The NGS assay used a 5% threshold for reporting low-frequency variants. The level of complete plus partial nucleotide sequence concordance between Sanger sequencing and NGS was 99.9%. Among the 138 protease/RT sequences, a mean of 6.4 DRMs was identified by both Sanger and NGS, a mean of 0.5 DRM was detected by NGS alone, and a mean of 0.1 DRM was detected by Sanger sequencing alone. Among the 39 integrase sequences, a mean of 1.6 DRMs was detected by both Sanger sequencing and NGS and a mean of 0.15 DRM was detected by NGS alone. Compared with Sanger sequencing, NGS estimated higher levels of resistance to one or more antiretroviral drugs for 18.2% of protease/RT sequences and 5.1% of integrase sequences. There was little evidence for technical artifacts in the NGS sequences, but the G-to-A hypermutation was detected in three samples. In conclusion, the IVD NGS assay evaluated in this study was highly concordant with Sanger sequencing. At the 5% threshold for reporting minority variants, NGS appeared to attain a modestly increased sensitivity for detecting low-frequency DRMs without compromising sequence accuracy.

KEYWORDS antiviral drug resistance, human immunodeficiency virus, integrase, mutations, next-generation sequencing, proteases, reverse transcriptase

Two factors motivate the increased use of next-generation sequencing (NGS) for diagnosing HIV-1 drug resistance in both research and clinical settings. First, the cost of NGS can be considerably lower than that of dideoxynucleotide Sanger sequencing should a sufficient number of samples be tested in the same sequencing run (1, 2). Second, the ability of NGS to detect low-frequency drug resistance mutations (DRMs) not detected by Sanger sequencing has potential advantages for improved patient outcomes (3).

However, Sanger sequencing has been used for 2 decades to diagnose HIV-1 drug resistance and has been shown to be highly reproducible and interpretable in clinical settings. In contrast, NGS technologies have been rapidly evolving, resulting in changes to both laboratory and bioinformatics analysis protocols (4). Moreover, no *in vitro* diagnostic (IVD) assay for HIV-1 genotypic resistance testing using NGS has been approved by the U.S. Food and Drug Administration (FDA). In this study, we compared

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the performance of an IVD NGS assay for HIV-1 genotypic resistance testing with the current standard approach using direct PCR Sanger sequencing.

MATERIALS AND METHODS

Patients and samples. We selected remnant cryopreserved plasma samples from individuals undergoing Sanger sequencing at the Stanford University Diagnostic Virology Laboratory for a blind comparison with the Vela Diagnostics Sentosa SQ HIV genotyping NGS assay. We selected samples that by Sanger sequencing had many common nucleoside reverse transcriptase (RT) inhibitor (NRTI)-, nonnucleoside RT inhibitor (NNRTI)-, protease (PR) inhibitor (PI) resistance-, and integrase (IN) strand transfer inhibitor (INSTI)-associated DRMs. Samples were obtained between 2001 and 2016 and were selected if their plasma HIV-1 RNA level (viral load [VL]) was ≥2.0 log copies/ml. The study was approved by the Stanford University Institutional Review Board.

Dideoxynucleoside Sanger sequencing. Dideoxynucleoside PCR Sanger sequencing for patient management was performed separately for PR/RT and IN sequences. The PR/RT sequences encompassed the entire PR and RT codons 1 to 300. IN sequences encompassed the entire IN. Plasma samples (400 μl) were ultracentrifuged, and the pellet was subjected to RNA extraction and SuperScript III one-step RT-PCR (Thermo Fisher Scientific) followed by a second PCR. The complete set of primers for Sanger sequencing is listed in Table S1 in the supplemental material. Bidirectional sequencing was performed using BigDye Terminators (Thermo Fisher Scientific), with the products being resolved electrophoretically on an ABI 3730 sequencer. A mixture was defined as a position having a secondary peak that comprises at least 20% of the area under the curve and evidence of a mixture in both directions.

NGS. The Sentosa SQ HIV genotyping assay is a next-generation sequencing (NGS)-based integrated work flow, comprising kits for RNA extraction, HIV-1 library preparation, and sequencing; a robotic liquid-handling system for RNA extraction and library preparation; Ion Torrent's instruments for deep sequencing; and data analysis and reporting software. The Sentosa SQ HIV genotyping NGS assay work flow is highly automated and requires about 3.5 h of hands-on time with a total turnaround time of about 27 h. The assay processes up to 15 plasma samples (730 μl per sample) simultaneously. The system sequences the entire PR sequence, the first 376 amino acids of the RT sequence, and the entire IN sequence. In this study, the median sequencing coverage for PR/RT and IN was 3,243 reads (interquartile range [IQR], 1,585 to 6,409 reads). In its current form and for the purposes of this study, the assay exports a FASTA file containing a single consensus nucleotide sequence in which positions containing nucleotide mixtures with variants present at or above 5% are represented as IUPAC ambiguities.

Data analysis. Because many more samples underwent Sanger sequencing of the PR/RT than the IN sequence, we performed separate analyses for the PR/RT and IN sequences. Complete nucleotide concordance was defined as both Sanger sequencing and NGS identifying the same nucleotide or ambiguity code at a position. Partial nucleotide discordance was defined as one method identifying a nucleotide mixture and the other identifying one of the mixture's components. Complete nucleotide discordance was defined as both methods identifying different nonambiguous nucleotides or ambiguous nucleotides that were nonoverlapping.

For concatenated PR/RT sequences and for IN sequences, we submitted the Sanger sequencing and NGS FASTA files to the Stanford HIV Drug Resistance Database (HIVDB) genotypic resistance interpretation program and selected the "Sequence quality" and "Drug resistance" spreadsheet output options (5). We compared the rows in the "Sequence quality" spreadsheets generated from the Sanger sequencing and NGS sequences to determine concordance for detecting (i) mutations, defined as amino acid differences from the subtype B consensus amino acid sequence (https://www.hiv.lanl.gov/content/sequence/HIV/CONSENSUS/Consensus.html); (ii) DRMs, defined as mutations assigned a penalty score by the HIVDB interpretation system (5), with DRMs detected by Sanger sequencing and NGS being considered concordant if they were present regardless of what other mutations were also present; (iii) the number of signature APOBEC mutations, defined as mutations specific for APOBEC-mediated RNA editing, with three or more being associated with a high probability of G-to-A hypermutation (6); and (iv) the proportion of positions with highly unusual mutations, defined as having a prevalence of <0.01% in HIVDB (6) and not being a known DRM or signature APOBEC mutation.

We compared the rows in the "Drug resistance summary" spreadsheets generated from the Sanger sequencing and NGS sequences to determine the concordance for the detection of categorical drug resistance interpretations for the most commonly used antiretrovirals (ARVs): the NRTIs lamivudine (3TC) and emtricitabine (FTC) (which were treated as one drug), abacavir (ABC), zidovudine (AZT), and tenofovir (TDF); the NNRTIs efavirenz (EFV), etravirine (ETR), and rilpivirine (RPV); the PIs atazanavir (ATV), darunavir (DRV), and lopinavir (LPV); and the INSTIs dolutegravir (DTG), elvitegravir (EVG), and raltegravir (RAL). There were five predicted drug resistance interpretation levels: susceptible, potential low-level resistance, low-level resistance, intermediate resistance, and high-level resistance (5). These levels were used to compare the drug resistance interpretations reported by NGS and Sanger sequencing.

Accession number(s). The complete set of 138 PR/RT and 39 IN sequences determined by NGS and Sanger sequencing, annotated by sample identifier and sequencing method, has been submitted to GenBank and may be found under accession numbers KY190203, KY190186, KY190168, KY190161, and MG800345 to MG800626 (Data Set S3).

RESULTS

Patients and samples. Overall, 143 samples were sequenced by both NGS and Sanger sequencing, including 104 that underwent PR/RT sequencing, 34 that under-

went PR/RT and IN sequencing, and 5 that underwent just IN sequencing. Thus, 138 samples underwent PR/RT sequencing and 34 underwent IN sequencing by both Sanger sequencing and NGS. In all samples, both Sanger sequencing and NGS encompassed the entire PR sequence, the first 250 positions of the RT sequence, and the entire IN sequence. Twelve samples (8.4% of 143) had non-B subtypes, including subtypes C ($n = 4$), CRF01_AE ($n = 3$), A ($n = 2$), G ($n = 1$), CRF02_AG ($n = 1$), and CRF07_BC ($n = 1$). The VL was available for 127 (88.8%) of 143 samples. The median VL for these samples was 3.8 $\log_{10}$ copies/ml (IQR, 3.1 to 4.5 $\log_{10}$ copies/ml).

Treatment histories were available for 112 individuals undergoing PR/RT sequencing. Among these individuals, the median numbers of NRTIs, NNRTIs, and PIs were 4, 1, and 2, respectively. Among 29 individuals undergoing IN sequencing for whom the treatment history was available, the median number of INSTIs received was 1.

Table 1 lists each of the NRTI, NNRTI, PI, and INSTI DRMs detected by Sanger sequencing according to its frequency in the tested samples. The samples contained 137 distinct DRMs: 40 NRTI-, 34 NNRTI-, 41 PI-, and 22 INSTI-associated DRMs. Table S2 in the supplemental material lists the DRMs and their penalty scores for the most commonly used ARVs. There was a high correlation between the proportions of DRMs in this data set and those from ARV-treated persons in HIVDB ($r^2 > 0.8$; $P < 0.001$).

Nucleotide sequence concordance and sequence ambiguities. In both the PR/RT and IN sequences, the nucleotide sequences determined by Sanger sequencing and NGS were highly concordant: 98.37% of nucleotides were identical, 1.58% were partially discordant, and 0.05% were completely discordant. Not surprisingly, the median number of IUPAC ambiguities was significantly higher in the sequences determined by NGS than those determined by Sanger sequencing: 2.1 (IQR, 1.0 to 3.5) versus 1.1 (IQR, 0.4 to 1.8) ($P < 0.001$, Mann-Whitney U test). The median 1.1% level of IUPAC ambiguities detected by Sanger sequencing is similar to the proportions of 0.7%, 1.1%, and 1.1% reported from three laboratories performing Sanger sequencing on similar heavily treated patients (7, 8). The higher proportion of ambiguities by NGS results from its lower threshold for detecting variants.

Mutations, DRMs, and drug resistance interpretations. Among the 138 PR/RT sequences, a mean of 24.2 mutations, defined as differences from the subtype B amino acid consensus sequence, was detected by both Sanger sequencing and NGS (Table 2). NGS identified a mean of 4.4 mutations not detected by Sanger sequencing, whereas Sanger sequencing identified a mean of 0.6 mutation not detected by NGS ($P < 0.001$, Student's t test). Among the 39 IN sequences, a mean of 12.1 mutations was identified by both Sanger sequencing and NGS (Table 2). NGS identified a mean of 2.7 mutations not detected by Sanger sequencing, whereas Sanger sequencing identified a mean of 1.2 mutations not detected by NGS ($P = 0.03$, Student's t test).

Among the 138 PR/RT sequences, a mean of 6.4 drug resistance mutations (DRMs) was identified by both Sanger sequencing and NGS (Table 2). NGS identified a mean of 0.5 mutation not detected by Sanger sequencing, whereas Sanger sequencing detected a mean of 0.1 mutation not detected by NGS ($P < 0.001$, Student's t test). Among the 39 IN sequences, a mean of 1.6 DRMs was identified by both Sanger sequencing and NGS (Table 2). NGS identified a mean of 0.2 DRM detected by Sanger sequencing, whereas Sanger sequencing identified a mean of 0.03 DRM not detected by NGS ($P = 0.09$, Student's t test). Among the 11 DRMs detected by Sanger sequencing but not NGS, each was present as a mixture by Sanger sequencing and 6 were present below the 5% threshold when the raw NGS reads were examined.

On average, there was no significant difference between Sanger sequencing and NGS in the predicted levels of resistance to any of the NRTIs, NNRTIs, PIs, and INSTIs (Table 3). However, for the 138 PR/RT samples, NGS detected a higher level of NRTI resistance for 6.2% ($n = 9$) of samples, a higher level of NNRTI resistance for 11.2% ($n = 16$) of samples, and a higher level of PI resistance for 4.1% ($n = 6$) of samples than Sanger sequencing (Tables 4 to 6). In contrast, Sanger sequencing detected higher levels of NRTI, NNRTI, and PI resistance for 0.7% ($n = 1$), 1.4% ($n = 2$), and 0.7% ($n =$

TABLE 1 NRTI, NNRTI, PI, and INSTI DRMs detected by Sanger sequencing genotypic resistance testing in 138 PR/RT and 39 IN sequences[a]

Inhibitor and DRM	No.	%	% HIVDB
NRTI			
M184V	67	48.6	53.6
M41L	52	37.7	27.3
D67N	41	29.7	26
T215Y	38	27.5	24.6
L210W	31	22.5	16.6
K70R	29	21	17.6
K219Q	27	19.6	10.5
L74V	20	14.5	7.5
K65R	19	13.8	6
T215F	19	13.8	9.1
L74I	10	7.2	3.7
K219E	10	7.2	5.8
E44D	10	7.2	7.2
T69D	9	6.5	5.4
D67G	8	5.8	2.3
T69ins	7	5.1	0.7
A62V	7	5.1	4.5
T215D	5	3.6	0.5
K219R	4	2.9	2.4
M184I	4	2.9	2
K219N	4	2.9	2.6
Y115F	3	2.2	3
V75M	3	2.2	3.2
E44A	3	2.2	1.3
K70G	3	2.2	0.3
K70E	3	2.2	1
T215E	3	2.2	0.1
K70Q	2	1.4	0.3
E40F	2	1.4	0.5
V75I	2	1.4	2.8
F77L	2	1.4	1.7
D67H	2	1.4	0.2
T215L	1	0.7	0.1
D67E	1	0.7	0.4
T215I	1	0.7	1.3
Q151M	1	0.7	2.6
F116Y	1	0.7	2.1
T215A	1	0.7	0.1
V75T	1	0.7	1.1
T215C	1	0.7	0.5
NNRTI			
K103N	46	33.3	29.5
Y181C	28	20.3	16.9
K101E	17	12.3	6.9
G190A	14	10.1	13.5
V108I	14	10.1	7.7
P225H	12	8.7	4.2
H221Y	11	8	6.7
L100I	9	6.5	3.4
A98G	8	5.8	6.4
K101P	7	5.1	1.1
K238T	6	4.3	1.9
Y188L	6	4.3	3.7
Y181I	4	2.9	0.7
E138A	4	2.9	3.3
G190S	4	2.9	2
Y188F	3	2.2	0.1
V179F	3	2.2	0.2
K101H	2	1.4	0.9
Y188C	2	1.4	0.6
E138K	2	1.4	0.5
G190E	2	1.4	0.4
K238N	2	1.4	0.3

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TABLE 1 (Continued)

Inhibitor and DRM	No.	%	% HIVDB
F227L	2	1.4	2.7
K103S	2	1.4	1.1
M230L	2	1.4	1.7
Y181F	1	0.7	0.1
Y188H	1	0.7	0.3
V179L	1	0.7	0.1
E138Q	1	0.7	1.1
V106A	1	0.7	1.2
V179E	1	0.7	1.1
V106M	1	0.7	5.1
L100V	1	0.7	0.1
V179D	1	0.7	2.4
PI			
L90M	27	19.6	25.8
M46I	19	13.8	19.7
V82A	17	12.3	20.5
L10F	14	10.1	7.6
I54V	14	10.1	21.8
L33F	11	8	11.5
K43T	10	7.2	0.1
I84V	9	6.5	1
N88D	9	6.5	4.9
D30N	8	5.8	5.3
V32I	6	4.3	4.6
I47V	6	4.3	0.1
G73S	5	3.6	6.6
L23I	5	3.6	1.3
M46L	5	3.6	8
L89V	4	2.9	3.2
V82T	4	2.9	2.1
G48V	3	2.2	2.8
V11I	3	2.2	2.7
F53L	3	2.2	5.6
I54M	3	2.2	1.9
I50V	3	2.2	1.3
I54L	3	2.2	2.6
G73T	3	2.2	1.9
K20T	2	1.4	0.1
G73A	2	1.4	0.4
L76V	2	1.4	3.5
Q58E	2	1.4	6.3
N88S	2	1.4	1.5
V82S	2	1.4	0.9
T74P	2	1.4	1.8
I54S	1	0.7	0.5
L24I	1	0.7	5.2
I50L	1	0.7	0.1
I54T	1	0.7	0.6
G48M	1	0.7	0.4
I47A	1	0.7	0.5
V82M	1	0.7	0.4
I54A	1	0.7	1
L24M	1	0.7	0.1
V82F	1	0.7	1.6
INSTI			
N155H	11	28.2	29.9
G140S	5	12.8	24.6
T97A	5	12.8	12.2
E92Q	5	12.8	6
Q148R	5	12.8	7.1
Q148H	4	10.3	22
E138K	4	10.3	0.5
G163K	3	7.7	1.7
G163R	2	5.1	7

(Continued on next page)

TABLE 1 (Continued)

Inhibitor and DRM	No.	%	% HIVDB
G140A	2	5.1	1.7
E138A	2	5.1	3.3
E157Q	2	5.1	7.4
Y143R	2	5.1	6.6
Y143C	2	5.1	3.9
S147G	2	5.1	1.5
T66I	1	2.6	1.2
L74M	1	2.6	0
L74I	1	2.6	3.7
Q95K	1	2.6	1.4
S230R	1	2.6	3.5
T66A	1	2.6	0.5
Q148N	1	2.6	0.2

[a]No., number of DRMs among the clinical samples; %, percentage of the samples containing the DRM; % HIVDB, prevalence of the DRM among ARV-treated individuals in the Stanford HIV Drug Resistance Database.

1) of samples, respectively. Overall, 26 (18.2%) individuals with PR/RT sequences had a higher level of predicted resistance to one or more RTIs or PIs based on NGS. Four (2.8%) had a higher level of predicted resistance based on Sanger sequencing. For the 39 IN samples, NGS detected a higher level of INSTI resistance for 5.1% ($n = 2$) of the samples (Table 7).

Quality control comparison. There was no significant difference in the mean number of signature APOBEC mutations per sequence between Sanger sequencing and NGS in either PR/RT sequences (0.02 for Sanger sequencing versus 0.14 for NGS; $P = 0.2$, paired Student's *t* test) or IN sequences (0.05 for Sanger sequencing versus 0.41 for NGS; $P = 0.06$). One PR/RT sequence and three IN sequences determined by NGS had three or more signature APOBEC mutations. Sample B2-11 had 12 signature APOBEC mutations, including six stop codons. Samples B3-25, B1-33, and B3-02 had five, four, and three signature APOBEC mutations, respectively. In contrast, no sequence determined by Sanger sequencing had more than two signature APOBEC mutations.

TABLE 2 Mean number of amino acid mutations and DRMs detected by standard Sanger sequencing genotypic resistance testing and NGS[a]

Gene and mutation category[b]	Mean value by:		*P* value[c]
	Sanger sequencing	NGS	
PR/RT ($n = 138$ sequences)			
No. of mutations			
Shared	24.20	24.20	NA
Unique	0.64	4.38	<0.001
No. of DRMs			
Shared	6.39	6.39	NA
Unique	0.10	0.54	<0.001
IN ($n = 39$ sequences)			
No. of mutations			
Shared	12.05	12.05	NA
Unique	1.18	2.69	0.03
No. of DRMs			
Shared	1.59	1.59	NA
Unique	0.03	0.15	0.09

[a]Amino acid mutations are defined as differences from the subtype B consensus reference sequence. Drug resistance mutations (DRMs) are mutations that receive a penalty score in the HIVDB drug resistance interpretation program.
[b]Shared mutations and DRMs are those that were detected by both NGS and Sanger sequencing. Unique mutations and DRMs are those that were detected by either NGS or Sanger sequencing but not by both.
[c]*P* values were determined by Student's *t* test. NA, not applicable.

TABLE 3 Mean levels of predicted reduced ARV susceptibility of the 138 PR/RT and 39 IN samples according to genotypic resistance testing by Sanger sequencing versus NGS

Drug class	Drug[a]	Mean susceptibility level[b] ± SD		*P* value[c]
		Sanger sequencing	NGS	
NRTIs (n = 138 sequences)	3FTC	3.43 ± 1.79	3.46 ± 1.79	0.9
	ABC	3.80 ± 1.57	3.78 ± 1.60	0.9
	AZT	3.16 ± 1.86	3.13 ± 1.83	0.9
	TDF	3.12 ± 1.58	3.12 ± 1.61	1.0
NNRTIs (n = 138 sequences)	EFV	3.33 ± 1.86	3.41 ± 1.86	0.7
	ETR	2.54 ± 1.61	2.64 ± 1.64	0.6
	RPV	2.94 ± 1.85	3.06 ± 1.88	0.3
PIs (n = 138 sequences)	ATV/r	2.23 ± 1.69	2.26 ± 1.70	0.9
	DRV/r	1.43 ± 1.03	1.43 ± 1.05	1.0
	LPV/r	2.09 ± 1.58	2.12 ± 1.59	0.9
INSTIs (n = 39 sequences)	DTG	2.31 ± 1.25	2.33 ± 1.27	0.9
	EVG	3.77 ± 1.70	3.79 ± 1.71	0.9
	RAL	3.79 ± 1.67	3.79 ± 1.67	1.0

[a]3FTC, lamivudine and emtricitabine; ABC, abacavir; AZT, zidovudine; TDF, tenofovir; EFV, efavirenz; ETR, etravirine; RPV, rilpivirine; ATV/r, ritonavir-boosted atazanavir; DRV/r, ritonavir-boosted darunavir; LPV/r, ritonavir-boosted lopinavir; DTG, dolutegravir; EVG, elvitegravir; RAL, raltegravir.
[b]Susceptibility levels were given the following scores: 1 for susceptible, 2 for potential low-level resistance, 3 for low-level resistance, 4 for intermediate resistance, and 5 for high-level resistance.
[c]*P* values were determined by Student's *t* test.

The PR/RT sample B2-11, which had 12 signature APOBEC mutations, including six stop codons at the default 5% threshold, had no signature APOBEC mutations or stop codons at a 20% threshold. This sample had two NNRTI resistance mutations, G190E and M230I, which are among the few NNRTI resistance mutations that can be caused by APOBEC (Table 5). G190E was detected by both Sanger sequencing and NGS, but M230I was detected only by NGS (at the 5% threshold but not the 20% threshold). This sample had a VL of 3.8 log copies/ml and a subtype B virus.

The mean number of highly unusual mutations per sequence in PR/RT was higher for the sequences determined by NGS than for the sequences determined by Sanger sequencing (0.75 versus 0.29; $P = 0.002$, paired Student's *t* test). There was no significant difference in the mean number of highly unusual mutations in the IN sequences (0.44 versus 0.38; $P = 0.8$, paired Student's *t* test). Among the 138 PR/RT sequences, the highest number of highly unusual mutations was four for Sanger sequencing. For NGS, five samples had a higher number of highly unusual mutations, including one with six and four with five highly unusual mutations.

DISCUSSION

This study is one of the largest comparisons of HIV-1 genotypic resistance testing conducted using Sanger sequencing and NGS and one of the few comparing Sanger sequencing to an IVD NGS assay. Despite the use of different methods for sample preparation and amplification, the level of complete plus partial nucleotide sequence concordance between Sanger sequencing and NGS was extraordinarily high, matching the 99.9% concordance rate reported in a previous study comparing different Sanger sequencing protocols (8). Compared with Sanger sequencing, NGS detected significantly more DRMs per sequence and estimated higher levels of resistance to one or more ARVs for 18.2% of PR/RT sequences and 5.1% of IN sequences.

Sanger sequencing usually detects HIV-1 variants present in proportions above 20%, with a range of 10% to 30%, depending on the nucleotide context (7, 9–12). In contrast, the proportion at which variants can be detected by NGS depends on the selected threshold. Although detection thresholds as low as 1% have often been used in research studies, many of these studies excluded samples with low plasma virus levels (13–17). Two recent descriptions of HIV-1 NGS analysis pipelines suggested using a detection threshold of 5% to minimize technical artifacts (18, 19).

TABLE 4 NRTI resistance mutations and estimated NRTI resistance levels for sequences with differences in genotypic resistance testing results by Sanger sequencing and NGS

Sample group and sample identifier	DRM detected by:			Sanger sequencing score → NGS score[a]			
	Sanger sequencing and NGS	Sanger sequencing alone	NGS alone	3FTC	ABC	AZT	TDF
Samples for which NGS reported higher levels of resistance to ≥1 NRTIs							
B3-31	41L, 215Y		184V	1 → 5	4	5 → 4	3
B3-10	184V		65R, 74V	5	3 → 5	1	1 → 4
B3-28	41L, 210W, 215Y		67N	3	5	5	4 → 5
B1-10	41L, 44D, 184V, 210W, 215Y		67N	5	5	5	4 → 5
B2-05	40F, 41L, 74V, 210W, 215Y, 219Q	67N	69ins	3 → 4	5	5	5
B3-25	65R, 184V		70E	5	5	1	4 → 5
B3-02	41L, 67N, 70R, 184V, 219E		215I	5	5	5	3 → 4
B2-17	184V		41L, 62V	5	3	1 → 2	1
B1-51	65R, 67N, 184V, 219E		70R, 215I	5	5	1 → 5	5
Samples for which Sanger sequencing reported higher levels of resistance to ≥1 NRTIs, B2-15	67N, 215I, 219E	70R		2 → 1	4 → 3	5 → 4	4 → 3
Samples for which Sanger sequencing or NGS detected different DRMs but for which levels of NRTI resistance were the same							
B1-42	41L, 62V, 184V, 210W, 215Y		219R	5	5	5	4
B2-31	67N, 219Q	215L	215V	1	3	4	3
B2-01	67N, 70R, 184V, 215Y, 219Q		74I	5	5	5	4
B2-38	41L, 69ins, 210W, 215Y		62V	4	5	5	5
B2-16	40F, 41L, 67N, 75 M, 210W, 215Y		44A, 75I, 219R	3	5	5	5
B2-04	67N, 70R, 219Q	215F	215V	2	4	5	4
B1-44	65R, 74I, 184V, 219R	70Q		5	5	1	5
B1-18	74V, 184V		219R	5	5	1	1

[a]Predicted levels of drug resistance according to the HIVDB genotypic resistance interpretation system, which were scored as follows: 1 for susceptible, 2 for potential low-level resistance, 3 for low-level resistance, 4 for intermediate resistance, and 5 for high-level resistance. For an additional 5 sequences, NGS detected two mutations at a DRM position for which Sanger sequencing detected a single mutation. However, the additional mutation did not result in a change in the estimated level of drug resistance. 3FTC, lamivudine and emtricitabine; ABC, abacavir; AZT, zidovudine; TDF, tenofovir.

Because of its extraordinary sensitivity, NGS is prone to two types of artifacts that occur uncommonly during Sanger sequencing: PCR error and APOBEC-mediated G-to-A hypermutation. PCR errors commonly occur during nested PCR but are rarely present at levels high enough to be detected by Sanger sequencing (20–23). In contrast, PCR errors are detected by NGS if the detection threshold is set too low. Indeed, if the number of amplifiable cDNA templates is low due to a low VL and/or inefficient RNA extraction, reverse transcription, or PCR, then much of the observed variability in an NGS sequence will represent PCR error (21, 22, 24). Indeed, studies using the Primer ID approach, which adds a unique label to each cDNA molecule, shows that the depth of NGS does not necessarily lead to the reliable detection of low-frequency variants (21, 25, 26).

NGS is also more likely than Sanger sequencing to detect low-level APOBEC-mediated G-to-A hypermutation, particularly in samples contaminated with proviral DNA (27–29). Hypermutated viruses are unlikely to be functional and have not been shown to contribute to virological failure (27–30). There are 13 DRMs that can be caused by APOBEC-mediated hypermutation, including D30N, M46I, and G73S in PR; D67N, E138K, M184I, G190SE, and M230I in RT; and E138K, G118R, and G163KR in IN. Determining whether a sequence contains an APOBEC-mediated G-to-A hypermutation is necessary for interpreting the significance of these DRMs.

We have hypothesized that an inappropriately low detection threshold for a sample

TABLE 5 NNRTI resistance mutations and estimated NNRTI resistance levels for sequences with differences in genotypic resistance testing results by Sanger sequencing and NGS

Sample group and sample identifier	DRM detected by:			Sanger sequencing score → NGS score[a]		
	Sanger sequencing and NGS	Sanger sequencing alone	NGS alone	EFV	ETR	RPV
Samples for which NGS reported higher levels of resistance to ≥1 NNRTIs						
B2-16			103N	1 → 5	1	1
B2-35	138A		103N	1 → 5	2	3
B3-33	103N		181C	5	1 → 4	1 → 4
B1-49	101E		190S	3 → 5	3 → 4	4 → 5
B3-31	181I		103N	4 → 5	5	5
B2-07	101H, 188L, 238T		98G, 179D, 181C, 190A	5	3 → 5	5
B1-51	181C		98G, 101E	4 → 5	4 → 5	4 → 5
B3-26	103N, 181C		190A, 238T	5	4	4 → 5
B1-43	190S		101E	5	2 → 4	3 → 5
B2-11[b]	190E		230I	5	4 → 5	5
B1-35	103N, 181C		221Y	5	4	4 → 5
B3-34	103N, 179L		181C	5	2 → 4	3 → 5
B1-33	103N, 238N		V179L	5	1 → 2	1 → 3
B2-01	181C, 188H, 190A		V108I, H221Y	5	4 → 5	5
B1-44	103N, 108I, 225H		221Y	5	1 → 2	1 → 3
B1-16	103N, 181C, 188C		106A, 190A	5	4	4 → 5
Samples for which Sanger sequencing reported higher levels of resistance to ≥1 NNRTIs						
B3-29	101E, 190A	103N, 181C		5	5 → 4	5
B1-37	181C	101E, 103N	221Y	5 → 4	4	5
Samples for which Sanger sequencing or NGS detected different DRMs but for which levels of NNRTI resistance were the same						
B2-32	100I, 238T	103N		5	4	5
B3-42	188L		103N	5	2	5
B3-36	98G, 101E, 103N, 190A		225H	5	4	5
B2-28	179E, 188L	108I		5	3	5
B2-29	98G, 103N, 108I, 221Y, 227L		238N	5	3	4
B1-04	100I, 103N, 225H		108I	5	4	5
B2-49	101P, 230L, 238N		101E, 138K, 181C	5	5	5

[a]Predicted levels of drug resistance according to the HIVDB genotypic resistance interpretation system, which were scored as follows: 1 for susceptible, 2 for potential low-level resistance, 3 for low-level resistance, 4 for intermediate resistance, and 5 for high-level resistance. EFV, efavirenz; ETR, etravirine; RPV, rilpivirine.

[b] Sample B2-11 had evidence for an APOBEC-mediated G-to-A hypermutation. Both G190E (which was also detected by Sanger sequencing) and M230I (which was detected only by NGS) are DRMs that occur in the APOBEC dinucleotide context.

can be determined in a *post hoc* analysis by counting its number of highly unusual and signature APOBEC mutations at different thresholds (6, 31). The presence of a large number of highly unusual mutations above a threshold suggests that the threshold is too low and that some of the low-frequency mutations are PCR errors. Likewise, the presence of a large number of signature APOBEC mutations above a threshold suggests that those DRMs that can be caused by APOBEC-mediated hypermutation may not reflect ARV selection pressure.

In this study, we identified one PR/RT sample and three IN samples with evidence for an APOBEC-mediated G-to-A hypermutation and five PR/RT samples with a slightly greater number of highly unusual mutations by NGS than by Sanger sequencing. The one hypermutated PR/RT sample was readily identifiable, and our *post hoc* analysis showed that a cutoff of 20% was appropriate for this sample. The relatively infrequent detection of signature APOBEC mutations and highly unusual mutations by NGS in this study is consistent with the conservative 5% threshold.

TABLE 6 PI resistance mutations and estimated PI resistance levels for sequences with differences in genotypic resistance testing results by Sanger sequencing and NGS

Sample group and sample identifier	DRM detected by:			Sanger sequencing score → NGS score[a]		
	Sanger sequencing and NGS	Sanger sequencing alone	NGS alone	ATV/r	DRV/r	LPV/r
Samples for which NGS reported higher levels of resistance to ≥1 PIs						
B3-31	10F, 82A		46L	3 → 4	1	4
B1-10	46L, 82A, 84V, 90M		46I, 54M	5	3 → 4	5
B1-42	46I, 90M		10F	4	1	3 → 4
B2-02	33F, 50V, 89V		46I	1 → 3	4	4
B3-28			90M	1 → 3	1	1 → 3
B2-04	88S		10F, 46I	5	1	1 → 3
Samples for which Sanger sequencing reported higher levels of resistance to ≥1 PIs, B3-19		46L		2 → 1	1	2 → 1
Samples for which Sanger sequencing or NGS detected different DRMs but for which levels of PI resistance were the same						
B1-07	10F, 11I, 32I, 46I, 54 M, 73A, 84V, 90M		47V	5	5	5
B2-32	48V, 54S, 82A		53L	5	1	5

[a]Predicted levels of drug resistance according to the HIVDB genotypic resistance interpretation system, which were scored as follows: 1 for susceptible, 2 for potential low-level resistance, 3 for low-level resistance, 4 for intermediate resistance, and 5 for high-level resistance. For an additional two sequences, NGS detected two mutations at a DRM position for which Sanger detected a single mutation. However, the additional mutation did not result in a change in the estimated level of drug resistance. ATV/r, ritonavir-boosted atazanavir; DRV/r, ritonavir-boosted darunavir; LPV/r, ritonavir-boosted lopinavir.

Low-frequency DRMs include those that were once dominant in an individual's virus population as a result of transmitted or acquired resistance but that, in the absence of drug pressure, were outgrown by more fit variants (32–34) and those that have emerged under selective drug pressure but that have not reached levels detectable by Sanger sequencing (27, 35). In this study of samples obtained primarily from heavily treated individuals with multiple DRMs detectable by Sanger sequencing, NGS detected

TABLE 7 INSTI resistance mutations and estimated INSTI resistance levels for sequences with differences in genotypic resistance testing results by Sanger sequencing and NGS

Sample group and sample identifier	DRM detected by:			Sanger sequencing score → NGS score[a]		
	Sanger sequencing and NGS	Sanger sequencing alone	NGS alone	DTG	EVG	RAL
Samples for which NGS reported higher levels of resistance to ≥1 INSTIs						
B3-17	148H		140S	3 → 4	5	5
B3-41	97A, 138A, 143C		155H	3	4 → 5	5
Samples for which Sanger sequencing or NGS detected different DRMs but for which levels of INSTI resistance were the same						
B1-33[b]	138A, 140A, 148R		163R	5	5	5
B3-07	155H		157Q	2	5	5
B3-09	155H, 163K	97A		2	5	5
B3-25[b]	155H		97A, 163K	2	5	5

[a]Predicted levels of drug resistance according to the HIVDB genotypic resistance interpretation system, which were scored as follows: 1 for susceptible, 2 for potential low-level resistance, 3 for low-level resistance, 4 for intermediate resistance, and 5 for high-level resistance. DTG, dolutegravir; EVG, elvitegravir; RAL, raltegravir.
[b]Samples B1-33 and B3-25 had weak evidence for an APOBEC-mediated G-to-A hypermutation. G163R and G163K are accessory INSTI resistance mutations that occur in the APOBEC dinucleotide context.

4.4 additional PR/RT mutations per sample, of which 0.5 were DRMs, and 2.7 additional IN mutations per sample, of which 0.2 were DRMs. The number of additional DRMs detected by NGS than by Sanger sequencing was higher in several other NGS studies of treated individuals that used detection thresholds lower than the threshold used in this study (13, 16, 36, 37).

The strongest evidence for the clinical significance of low-frequency DRMs has been for the CXCR5 inhibitor (38) and NNRTI (39, 40) classes. One meta-analysis and two subsequent case-control and cohort studies have shown that low-frequency NNRTI DRMs are associated with an increased risk of virological failure on a first-line NNRTI-containing regimen (15, 39, 41). The cohort study showed an increased risk of virological failure associated with low-frequency variants present at levels above 5% but not at lower levels (41). Low-frequency accessory PI-associated DRMs have not been shown to reduce the response to a first-line ritonavir-boosted ATV- or ritonavir-boosted DRV-containing regimen (42).

Low-frequency DRMs have been associated with an increased risk of virological failure in one retrospective salvage therapy study (13) but demonstrated only a trend toward increased virological failure in a second study of salvage therapy with the potent combination of ritonavir-boosted darunavir, raltegravir, and etravirine (37). Low-frequency DRMs also appear to be more likely to be clinically significant in patients with a treatment history compatible with the DRM, possibly because in this setting the low-frequency DRMs are less likely to be artifactual and more likely to be linked to compensatory mutations (16, 36, 40).

In conclusion, this study demonstrates a high concordance rate between NGS and Sanger sequencing. Despite using a conservative 5% threshold for low-frequency variants, NGS still detected a mean of an additional 0.5 PR/RT DRM and 0.1 IN DRM per sequence, resulting in a predicted increased level of resistance to one or more ARVs for 18% of PR/RT sequences and 5% of IN sequences. Additional studies are needed to elucidate the clinical significance of different low-frequency DRMs, although it is likely that those DRMs causing the greatest reductions in susceptibility to ARVs with low genetic barriers to resistance will be the most clinically relevant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00105-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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