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Blinded, Multicenter Comparison of Methods To Detect a Drug-Resistant Mutant of Human Immunodeficiency Virus Type 1 at Low Frequency

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We determined the abilities of 10 technologies to detect and quantify a common drug-resistant mutant of human immunodeficiency virus type 1 (lysine to asparagine at codon 103 of the reverse transcriptase) using a blinded test panel containing mutant–wild-type mixtures ranging from 0.01% to 100% mutant. Two technologies, allele-specific reverse transcriptase PCR and a Ty1HRT yeast system, could quantify the mutant down to 0.1 to 0.4%. These technologies should help define the impact of low-frequency drug-resistant mutants on response to antiretroviral therapy.

The high replication rate and error-prone reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) generates a large population of genetically distinct variants (1). Variants with a fitness advantage can rapidly outcompete others (1). In the case of antiretroviral therapy, outgrowth of drug-resistant mutants can lead to treatment failure (7). After the removal of drug selection, resistant mutants often decline to undetectable levels, albeit at variable rates (2, 6). These “undetectable” mutants can rapidly reappear after reinitiation of antiretroviral therapy, but it is uncertain how often they cause treatment failure (3, 8, 12). Answering this question requires methods to accurately detect drug-resistant mutants present at a low frequency.

Standard genotyping methods provide a composite of the HIV sequences present, but they generally do not detect mutants that comprise less than 20% of the virus population (11, 18). Several assays have been developed with improved sensitivity for resistant mutants (4), 10, 13–17, 20); however, their relative performances have not been assessed. In the current study, a panel of wild-type–mutant HIV-1 mixtures was created to evaluate the performance of these assays. The mutant virus encodes the K103N mutation in HIV-1 RT that is commonly selected for by nonnucleoside RT inhibitor (NNRTI) therapy and confers cross-resistance to all FDA-approved NNRTIs.

Viral stocks were generated by CaPO4 transfection of 293T cells (9) with the infectious plasmid clones of HIV-1_LAI encoding wild-type 103K (AAA) or mutant 103N (AAC) RT (19). The HIV-1 RNA concentrations of stocks were determined (AmpliScor HIV-1 Monitor Assay version 1.0; Roche Molecular Systems, Branchburg, NJ). Viral mixtures were generated by adding appropriate amounts of mutant and wild-type virus stocks, as determined by the HIV-1 RNA concentration, to yield final mutant fractions of 0, 0.01, 0.1, 0.4, 1, 2, 5, 10, 25, 50, and 100%. Each of the viral mixtures was then spiked into 25 ml of HIV-seronegative human plasma from a single donor to obtain a final HIV-1 RNA concentration of ~1 × 106 RNA copies/ml. The mean (± standard deviation) HIV-1 RNA in the mixtures was 5.4 ± 9.2 × 105 copies/ml (each mixture was tested in triplicate), which is within twofold of the expected concentration.

A broad solicitation of interest in testing the panel was sent to academic, government, and industry laboratories working on the detection of minor drug-resistant variants. Thirteen laboratories were selected with the goal of maximizing the number of technologies evaluated. The technologies included pyrosequencing of PCR products (15), real-time allele-specific RT-PCR (ASPCR) (10, 13, 17), oligonucleotide ligation-based assays (OLA) (4), a Ty1/HIV-1 RT hybrid system (TyHRT)
Of using TyHRT is the ability to directly confirm the mutant phisms that can destabilize primer binding (6). An advantage allele analysis and false negatives from nucleotide polymor-
sitive, which may be attributable to differences in primer design 
testing methods. The third ASPCR method (10) was less sen-
sensitive, quantifying the mutant to 0.1 to 0.4% (Table 1). This 
table 1 shows the relative performances of the methods. 
control was reported as negative. The quantification limit of an 
higher-mutant fractions were detected and that the 0% mutant 
were tested once because of their large size and limited supply. 
was included (HIV-1 TRUGENE [Bayer, Tarry-
standard commercial 
lyzing mutation linkages without the need for cloning (16), but 
quantitative below 2% mutant (Table 1) but were not 
ected by increasing the number of clones analyzed, but the 
practical.

Despite the high sensitivity of ASPCR, it is limited by single-
allele by sequencing individual yeast clones and/or to identify 
novel NNRTI resistance mutations (reference 14 and data not 
showed). A shortcoming of the TyHRT assay is that resistance 
to nucleoside analogs and protease inhibitors cannot be as-
Pyrosequencing and SGS (45 sequences/mixture) had inter-
mediated detection limits of 2% mutant (Table 1) but were not 
quantitative below 10% 103N. SGS has the advantage of analy-
zying mutation linkages without the need for cloning (16), but 
a high sensitivity for minor variants requires the sequencing 
of many genomes, e.g., 490 single genomes to detect a mutant 
at a 1% frequency with 99% certainty, which is currently im-
practical.

The TRUGENE and VirosSeq v2.0 genotyping methods 
quantified the 103N mutant at frequencies of 5 and 10%, 
respectively. The sensitivities of these products are higher than 
previously reported for population-based sequencing (11), 
which may be attributable to scrutiny of the 103 codon by the 
laboratories and a low background of plasmid-derived recom-
binant viruses. Results with other mutants may differ, and 
more importantly, neither method was developed or cleared 
for minor-variant detection.

Cloning and sequencing of RT-PCR products from a single 
or multiple cDNA reactions (VirosSeq) did not improve 
quantification of mutants over that of the standard commercial 
assays (11). This result was not unexpected, since only 25 
clones per reaction were sequenced. Sensitivity could be im-
proved by increasing the number of clones analyzed, but the 
labor and cost involved are prohibitive.

The OLA (4) and LiPA (20) assays (LiPA was used here 
with LiPAScan, an automated strip reader) detected the 103N 
mutant at frequencies between 2 and 10% (Table 1). One

<table>
<thead>
<tr>
<th>Expected % 103N</th>
<th>ASPCRa</th>
<th>TyHRT</th>
<th>Pyro Seq</th>
<th>TRUGENEa</th>
<th>OLAa</th>
<th>LiPAa</th>
<th>Cloning ScDNAa</th>
<th>Cloning McDNAa</th>
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<td></td>
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<td>Lab 3</td>
<td>Lab 1</td>
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<td>Lab 3</td>
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<td>Lab 2</td>
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<td>0</td>
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<td>100</td>
<td>89.7</td>
<td>&gt;97</td>
<td>100 100 100</td>
<td>100 100</td>
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</table>

a For each assay, the mutant detection limit is shown in boldface and the quantification limit is in italics.
b 0% 103N/100% wild type.
c Mix, mixture of wild type and mutant detected.
d ND, not determined.
e For ASPCR, laboratories 1, 2, and 3 used different methods.
f Pyro Seq. pyrosequencing.
g VirosSeq v2.0 platform from Applied Biosystems.
h TRUGENE platform from Bayer Diagnostics.
i For OLA, samples assayed by laboratory 1 were spiked with a plasmid standard and thus were quantified.
j VERSANT HIV-1 Resistance assay (LiPA) (Bayer Healthcare-Diagnostics).
k Cloning (TOPO cloning kit; Invitrogen) from a single cDNA reaction (ScDNA); 25 clones were derived and sequenced (Big Dye Terminator kit v3.1; Applied Biosystems) from each RT-PCR product.
l Cloning (TOPO cloning kit; Invitrogen) from multiple, independent cDNA and RT-PCR reactions (McDNA) (25 per test sample); one clone was derived and sequenced (Big Dye Terminator kit v3.1; Applied Biosystems) from each RT-PCR product.
laboratory performing OLA also used external standards to improve quantification but misclassified the wild-type (0%) control as having 2 to 5% 103N and thus could not detect below 10% mutant (Table 1).

Other comparative studies of genotypic assays for HIV-1 mutant detection have been performed (4, 5, 11, 15), but none has covered the breadth of technologies evaluated here. Additional panels of virus mixtures and clinical samples need to be tested to assess the potential impacts of different codons, viral heterogeneity, and operator experience on assay performance, as has been noted previously (6, 18). Nevertheless, our findings indicate that several assays are available to study the emergence, decay, and therapeutic significance of minor populations of drug-resistant HIV-1.

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