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Sensitive Oligonucleotide Ligation Assay for Low-Level Detection of Nevirapine Resistance Mutations in Human Immunodeficiency Virus Type 1 Quasispecies▽†

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This study has adapted the oligonucleotide ligation assay (OLA) to probe for low-level nevirapine (NVP) resistance mutations K103N and Y181C in the human immunodeficiency virus type 1 (HIV-1) population of infected mother-infant pairs from Uganda. When NVP is used to prevent perinatal transmission, NVP-resistant HIV-1 clones may be rapidly selected due to a low barrier for mutation and a relatively high level of fitness (compared to that of other drug-resistant HIV-1 clones). Monitoring for even a low frequency of NVP resistance mutations may help predict the success of subsequent treatment or warrant the use of another regimen to prevent transmission in a subsequent pregnancy. The standard OLA was optimized by using nonstandard bases in oligonucleotides to allow promiscuous base pairing and accommodate significant HIV-1 heterogeneity. Radiolabeled as opposed to fluorescently tagged oligonucleotides increased the sensitivity, whereas alteration of the template, oligonucleotides, salt, and thermostable DNA ligase concentrations increased the specificity for the detection of minority codons. This modified OLA is now capable of detecting mutants with the K103N or the Y181C mutation present in an HIV-1 population at a frequency of ~0.4% and is at least 10- to 30-fold more sensitive than the original protocol. A cohort of 19 Ugandan mothers who received NVP treatment perinatally were sampled 6 weeks postdelivery. Ten of 19 HIV-1 DNA samples extracted from peripheral blood mononuclear cells had a detectable K103N (0.5 to 44%) or Y181C (0.8 to 92.5%) mutation, but only one plasma HIV-1 RNA sample had a viral population with the Y181C mutation. These findings suggest that OLA is a robust, sensitive, and specific method for the detection of low-frequency drug resistance mutations in an intrapatient HIV-1 population.

Three types of antiretroviral drugs (ARVs) are now used for the treatment of human immunodeficiency virus type 1 (HIV-1) infections, but only reverse transcriptase (RT) inhibitors are readily available to the vast majority of HIV-1-infected individuals in the developing world. The treatment regimen of choice is a combination of a nonnucleoside RT inhibitor (almost exclusively nevirapine [NVP]) and two nucleoside RT inhibitors, i.e., zidovudine (AZT) or stavudine plus lamivudine or didanosine.

In addition to being the backbone of most treatment regimens, NVP is provided as a single dose to block the mother-to-child transmission (MTCT) of HIV-1 in developing countries. In the absence of antiretroviral therapy, the frequency of MTCT is approximately 25 to 48% (2, 36, 38), whereas the administration of a short course of AZT therapy near the end of gestation (7, 8, 49) or the administration of a single dose of NVP at labor can reduce the rate of perinatal transmission to less than 20% (22, 25, 32, 35, 37). Although NVP remains very effective in the prevention of MTCT (22, 32), the administration of even a single dose of NVP to HIV-infected mothers and their newborns can select for HIV-1 variants with NVP resistance mutations (8, 11–13, 15, 25, 30, 50). The use of NVP for both therapy and prophylaxis in developing countries has resulted in the rapid emergence of ARV resistance, which may hamper efforts in future anti-HIV treatment campaigns (4, 27).

HIV-1 has been categorized into 10 different subtypes that share approximately 85 to 90% sequence identity within subtypes and <85% sequence identity between subtypes (1). Intersubtype sequence variation is a formidable obstacle to genotypic drug resistance testing. In the Americas and Europe, subtype B is the most prevalent, whereas the predominant subtype in sub-Saharan Africa varies by country (1). Under drug pressure, a previously conserved amino acid may be changed to overcome drug effects. Although the rate of HIV-1 intersubtype diversity is high in general, different subtypes follow similar evolutionary routes and generally select for the same pattern of mutations for resistance to a particular antiretroviral drug. Of course, these HIV-1 drug-resistant clones typically preexist in the patient's HIV-1 quasispecies prior to treatment. Single-nucleotide polymorphisms (SNPs) proximal to that which confers drug resistance hamper efforts to generate general genotypic drug resistance assays, since the discrimination step usually involves enzymatic recognition of a DNA-DNA duplex that comprises an oligonucleotide and a patient-derived HIV sequence. Unrelated or compensatory SNPs which do not themselves confer drug resistance may

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induce duplex contortion, such that enzymatic recognition of the SNP of interest is inhibited.

Several mutations confer resistance to NVP (39, 43, 47), but the most common are K103N and Y181C in subtype B (5, 17, 51). Recent findings suggest that the less frequent subtype B NVP resistance mutations, i.e., G190A, Y188C, and V106M, may be more commonly selected in nonsubtype B HIV-1 isolates (14, 33, 40). A single transversion mutation from AAA or AAG (both of which encode lysine) to AAT or AAC results in the K103N mutation, whereas a single transition mutation from TAT to TGT is responsible for the Y181C mutation. The Y181C change may result in a slightly greater fitness cost than the K103N change (6), but it also confers a higher level of NVP resistance than K103N (39). Both NVP resistance mutations emerge quite rapidly and have minimal fitness costs compared to the reductions in fitness conferred by most nucleoside RT inhibitor and protease inhibitor resistance mutations (24, 41). In addition, the reversion of NVP resistance mutations in the absence of treatment appears to be slower than that with most drugs (12, 16). Even low levels of ARV resistance mutations in a patient's HIV-1 population can predict subsequent drug failure and full resistance with continued drug selective pressure (27). Considering the limited monitoring of treatment that occurs in the developing world, a rapid and inexpensive method for the detection of low-frequency SNPs may have the greatest impact in the developing world, where NVP is used as prophylaxis to prevent MTCT and where NVP is the backbone of highly active antiretroviral therapy.

Due to the cost, limited specificity, and low sensitivity of most diagnostic assays for SNPs, the number of NVP-treated patients harboring NVP SNPs may be grossly underestimated (26, 44). Direct DNA sequencing of cDNA from patient plasma or from peripheral blood mononuclear cell (PBMC) extracts, such as with the Trugene system (Bayer Diagnostics) or the ViroSeq system (Applied Biosystems), may be the most common method; but it is also the least sensitive method used to detect SNPs as minority base changes (>20 to 30%) (10, 20, 28, 44). Subcloning and sequencing of HIV-1 clones (>100) can counter this low sensitivity of bulk sequencing, but they are very costly. New approaches such as 454 pyrosequencing permit the rapid detection of SNPs in a relatively short sequence, but at an exorbitant cost (31). Heteroduplex tracking assays and restriction fragment length polymorphism analysis are less costly and relative simple methods for SNP detection, but they are also less sensitive than desirable (they detect 5 to 10% of the SNPs present in an HIV-1 population). Finally, the INNO-LiPA HIV-1 RT and PRO assays (Innogenetic, Ghent, Belgium) are oligonucleotide-based hybridization assays that can discriminate between wild-type and mutant sequences and that have the ability to detect low-frequency polymorphisms (21, 48). All of these approaches, however, have limited functionality in probing for drug resistance mutations in diverse HIV-1 genomes. The most sensitive methods for the detection of low-level SNPs usually involve PCR-based amplification and discrimination (allelic-specific PCR). Briefly, a high-fidelity, thermostable polymerase extends only primers whose 3′-terminal bases are complementary to the base which encodes drug resistance (45). However, even substitutions distal from the specific SNP can reduce oligonucleotide primer binding and create primer-template mismatches, which are poorly recognized by a high-fidelity polymerase. For this reason, most assays that use this methodology use primers with sequence identity to the HIV-1 target sequence (aside from the 3′-terminal bases that encode the SNP) and are not suitable for detection of drug resistance-associated SNPs across divergent HIV-1 subtypes (46). Unfortunately, none of these techniques are easily adopted in a resource-limited setting.

In 1995, Frenkel et al. described an oligonucleotide ligation assay (OLA) for detection of the presence of drug resistance mutations or SNPs in the HIV *pol* gene (19). This assay uses a thermostable, high-fidelity, template-dependent DNA ligase to join two oligonucleotides annealed to a denatured PCR product. A downstream "common" oligonucleotide is 5′ phosphorylated and 3′ biotinylated (19). The upstream "variable" oligonucleotide contains the complement to either the wild-type or the mutant base at the 3′ end and is modified on the 5′ end with one of two optically distinct fluorescent tags. Ligated products are captured on a streptavidin-coated 96-well plate and read with a fluorimeter. Although the detection of mutant present at a frequency of 3% was possible, this technique was not optimized to accommodate the significant genetic variability between HIV-1 isolates. We have modified this method to increase the sensitivity of detection of resistance mutations present at a low level to accommodate the significant genetic diversity of HIV-1 and to specifically detect low-level NVP resistance-associated SNPs (K103N and Y181C) in HIV-infected patients. To increase the sensitivity to less than 0.5%, we have tagged the upstream oligonucleotide at the 5′ end with α-^{32}P and optimized the cycling conditions and the ligase input, salt, template, and oligonucleotide concentrations. To address the diversity of HIV-1, nonstandard nucleotides were introduced into primers opposite the highly polymorphic sites in the HIV-1 sequences of different subtypes. These nonstandard bases had promiscuous base pairing or simply maintained normal base-stacking interactions but did not impair ligase recognition of the 4-nucleotide (nt) window at the oligonucleotide gap. The resulting methodology can detect extremely minority SNPs (a single base change in 1 of 200 DNA strands).

MATERIALS AND METHODS

Patients. To test the radioactive OLA, we used a small set of samples from HIV-infected Ugandan mothers recruited to a community-based Uganda observational study, as described previously (2). Briefly, 19 of 109 women who had received antiretroviral therapy to prevent perinatal HIV transmission as part of a Ugandan Ministry of Health UNICEF/Elizabeth Glazer Pediatric AIDS Foundation-funded pilot program were enrolled between June and October 2000. Each woman received therapy at one of three hospitals located in Kampala, Uganda. Treatment consisted of a single dose of 200 mg NVP (n = 61) at the onset of labor. Blood was drawn from the mothers at a mean of 38.5 weeks postpartum. The study was reviewed and approved by the Institutional Review Boards at Case Western Reserve University, Cleveland, OH, and by the AIDS Research Subcommittee in Uganda. Additional details about this cohort are provided elsewhere (2).

Sample preparation. PBMC DNA was isolated from patient blood by using a QIAGEN (Valencia, CA) Blood Mini kit. First-round PCR was performed with primers RTS1 (TAAACAATGGCCATTGACAGAAGA) and RTA9 (TAAATTTAGGAGTCTTTCCCCATA), as described previously (42). A second round of PCR was performed with primers RTS2 (TCAAAAATTGGGCCTGAAAATCCAT) and RTA8 (GCTATTAAGTCTTTTGATGGGTCAT) with the same cycling conditions to produce an 839-bp amplicon. PCR cleanup was performed with a QIAquick PCR purification kit (QIAGEN). All oligonucleotides were obtained from Invitrogen (Carlsbad, CA). The purified second-round PCR products were resolved by electrophoresis on a 0.7% agarose TBE (Tris-borate-EDTA) gel, visualized with ethidium bromide, and quantified by comparing the

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band intensity to the band intensities for known amounts of Bio-Rad Low Mass DNA ladder (Bio-Rad, Hercules, CA) by using one-dimensional gel analysis software (Eastman Kodak, New Haven, CT). The PCR product was then diluted to 1.0 ng/μl and 5.0 ng/μl. The amount of product was also confirmed by obtaining spectrophometry measurements at 260 nm.

Assay-positive controls were obtained by insertion of the product obtained by PCR of primary patient isolates with primers RTS2 and RTA8 into pCR2.1TOPO, which was used to transform *Escherichia coli*. Plasmids were isolated from liquid cultures of candidate colonies, and the sequences were verified by DNA sequencing. Plasmids containing mutant or wild-type inserts were amplified with RTS2 and RTA8. The PCR product concentration was estimated as described above, and a dilution series of the appropriate clonal PCR product was run in parallel with the experimental samples with every preparation of radioactive probe.

Probe labeling. One hundred picomoles of the upstream oligonucleotide was incubated at 37°C for 10 min with 40 U T4 polynucleotide kinase (Invitrogen), 100 pmol of 3,000 Ci/mmol [γ-^{32}P]ATP (Perkin-Elmer), and 20 μl 5× T4 PNK forward buffer (Invitrogen) in a 100-μl reaction mixture. The labeling reaction mixtures were then incubated at 65°C for 10 min and transferred to ice. Cold labeling reactions were extracted with 25:24:1 (vol/vol/vol) phenol-chloroform-isoamyl alcohol (Roche, Basel, Switzerland) and eluted from G-25 Sephadex columns (Amersham, Piscataway, NJ), according to the manufacturer's instructions.

OLA. For each reaction, 5 μl control or patient amplicon dilution, 1.5 pmol ^{32}P-radiolabeled upstream oligonucleotide, 1.5 pmol cold downstream oligonucleotide, and 2.5 U Ampligase DNA ligase (Epicenter Technologies, Madison, WI) were added together in a 12-μl reaction mixture containing 16.7 mM Tris-HCl (pH 8.3), 0.07% Triton X-100, 0.8 mM dithiothreitol, 10 mM KCl, 8.3 mM $MgCl_2$, and 0.83 mM NAD. The reactions were subjected to 30 cycles of 93°C for 30 s and 37°C for 4 min. The reactions were stopped by addition of 5 μl loading buffer containing 0.1 M EDTA, 0.1% Triton X-100, 25% formamide, 0.025% bromophenol blue, and 0.025% xylene cyanole in water. Three microliters of each reaction mixture was separated by 10% denaturing polyacrylamide gel electrophoresis (PAGE) for 2 h. The gels were dried at 80°C under vacuum for 1 h and exposed to film overnight.

Calculation of mutation frequency. The bands on the gel were quantitated by using a Molecular Imager FX apparatus and Quantity One software (Bio-Rad). A set of standards, including two negative controls and 10 dilutions of a positive control (prepared as described above), were run with every preparation of labeled probe. After correction for lane noise from the standard curve, the ligation efficiency (E) was defined by fitting to the following exponential:

$$M = A_1 \times [\exp(I/t_1)] + y_0 \quad (1)$$

where I is the corrected signal intensity, and M is the template mass. A_1 (vertical offset), t_1 (curve rise rate), and y_0 (minimum vertical asymptote) are the independent parameters of nonlinear curve fitting (OriginLab software; OriginLab Corp., Northampton, MA), none of which was constrained during successive iterations with a chi-squared tolerance of 10^{-9}. These values represent only parameters which relate the signal to the template input, and since they are not derived from ab initio equations for ligase kinetics, they are not biochemically significant. Calculation of the template copy number assumed an average base-pair mass of 660 Da and an amplicon of 839 bp corresponding to the second-round PCR primers RTS2 and RTA8. With these parameters, we calculated the copy number from the corrected signal given by 2.4 ng/μl negative control template. This value was subtracted from y_0, which served as a cutoff for subsequent calculations. This was repeated for each preparation of radiolabeled probe.

Two dilutions of the PCR products from the patient samples were used for each probe: 5 ng and 25 ng (the total amount in a 12-μl reaction mixture). After calculation of the ligation efficiency for each band (equation 1), we corrected for inhibition with the high template input. In the absence of inhibition by excess template, the signal given by the 5-ng input ($E_{5\ ng}$) should be exactly 1/5 that given by the 25-ng input ($E_{25\ ng}$), or

$$5 \times E_{5\ ng} = E_{25\ ng} \quad (2)$$

The larger of the quantities (either $5 \times E_{5\ ng}$ or $E_{25\ ng}$) was chosen, and then the copy number (C_x) of this was calculated from a standard curve of OLA performed with a known copy number of a defined template. In this way, the quantity C_x was calculated individually for every sample for every preparation of probe. The larger copy number was used for calculation of the mutant content according to the following equation:

$$\text{Percent/mutant} = 100 \times [(C_{\text{mutant}})/(C_{\text{mutant}} + C_{\text{wild type}})] \quad (3)$$

This represents the simplest case, such as at site 181, for which only one mutant probe and one wild-type probe was necessary to assess the common drug resistance polymorphism. For the other two sites (sites 70 and 103), we used a similar equation to account for synonymous codon probes.

Quantitation controls for OLA. HIV-1 RT genes from over 100 HIV-1 subtype A- and subtype D-infected patients were sequenced previously. The PCR products from the patient samples harboring K103N, Y181C, or wild-type sequences (patients MTN121, INN131, MTN148, MTN207, MTA308, and MTA333) were cloned into pCR2.1TOPO (Invitrogen), according to the manufacturer's instructions. Plasmids were purified by using a QIAGEN Spin Mini kit and were then sequenced by using primers RTS2 and RTA8 to confirm the presence of the wild-type sequence or drug resistance mutations. To produce quantification controls for OLA, the specified RT region was amplified from these clones by PCR and then quantified on a gel and diluted to 0.05, 0.1, 0.5, 0.6, 1.2, 2.4, 3.6, 5.0, 7.0, and 10.0 ng/μl (working concentrations). These products were then subjected to OLA, as described above.

Clonal analysis for OLA. The PCR products from HIV-1-infected patients with various percentages of K103N, Y181C, or wild-type codons were inserted into pCR2.1TOPO and transformed into chemically competent Top 10 cells, according to the manufacturer's instructions. Transformants were plated on LB agar dishes with 100 μg/ml ampicillin coated with 1.6 mg 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. The plated bacteria were incubated for 16 h at 37°C. Up to 20 plates were generated to estimate the frequency of a drug resistance mutation in each patient. Ten to 1,000 clones (according to the frequency of the drug resistance mutations from the bulk OLA) were picked and grown in 3-ml LB cultures containing ampicillin. Aliquots of 150 μl were removed from 20 3-ml cultures and pooled for plasmid purification by using the QIAGEN Spin Mini kit. When the estimated frequency of an NVP resistance mutation was approximately 0.1%, 50 pools of 20 clones were sampled and subject to OLA. Inserts from the pools were amplified by PCR with primers RTS2 and RTA8, as described above, and diluted to 1.0 ng/μl. Mutant detection by OLA was performed with diluted PCR products. Individual plasmids from the mutant-positive pools were isolated from the remaining culture volume. Another OLA for mutant detection was performed with all individual clones from the pool to discover which clones were positive. Mutant clones were then sequenced at Davis Sequencing (Davis, CA) on an ABI 3730 genetic analyzer by using Applied Biosystems BigDye Terminator (version 3.0) sequencing chemistry.

RESULTS

An OLA was modified to detect NVP resistance mutations present at very low levels within the HIV population of an infected individual. The modified radioactive OLA relied on the high-fidelity ligation of two oligonucleotides by a proprietary DNA ligase. We developed this assay to detect the two most common NVP HIV resistance mutations appearing in RT, K103N and Y181C. We have probed for K70R, which commonly emerges as the first AZT resistance mutation under drug selective pressure (41). Detection of K70R is of interest when the levels of NVP and AZT resistance mutations in the HIV quasispecies of untreated patients are compared. For each site of interest, we used two oligonucleotides designed to abut one another on the template strand such that the mutation of interest was placed inside the ligase "fidelity window," i.e., within 2 bases up- or downstream of the ligation site (Fig. 1). The upstream oligonucleotide, termed the "variable" oligonucleotide, has a discriminating 3′ nucleotide, while the downstream oligonucleotide is "common," i.e., complementary to both wild-type and mutant sequences. In all cases, the downstream oligonucleotide was 5′ phosphorylated to drive ligation when no mismatches were present in the fidelity window.

The thermostability of the DNA ligase allowed increased ligation with increasing numbers of annealing/denaturation cycles (10 to 40 cycles) (3, 9, 18, 19, 46, 50). Following this cycling reaction, the radiolabeled ~30-nt unligated upstream oligonu-

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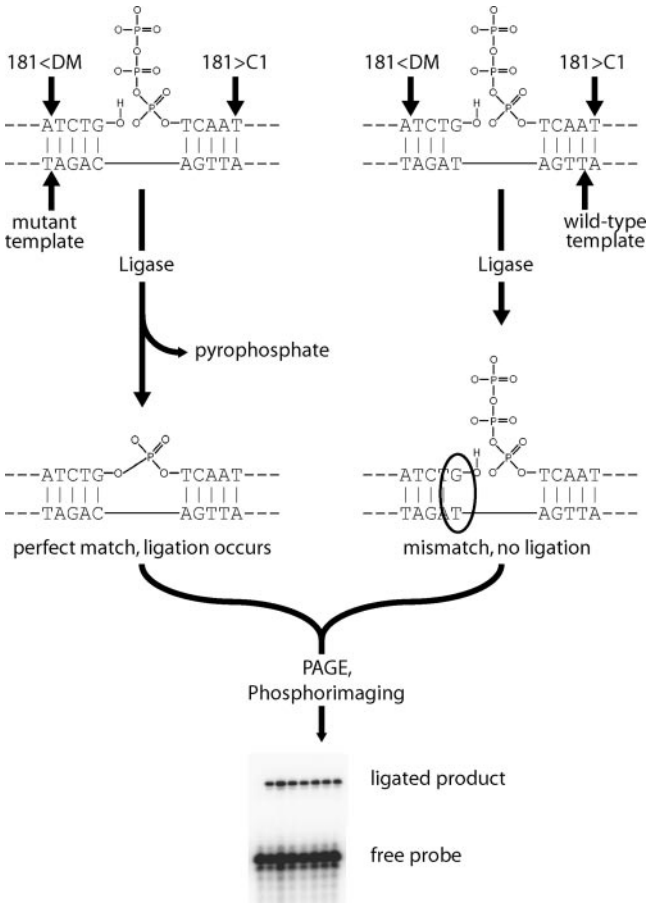


FIG. 1. Schematic of the oligonucleotide ligation assay. The discriminating upstream oligonucleotide (181<DM) and common downstream oligonucleotide (181>C1) are shown. Ligase is reactive only at a ligation-competent site, where perfect base pairing occurs both immediately upstream and downstream of the ligation-competent site. OLA products can be separated by denaturing PAGE (bottom).

cleotide was separated from the radiolabeled ~60-bp ligated product on a denaturing polyacrylamide gel (Fig. 1). Although a perfect match between each of the 4 bases inside the ligase fidelity window is necessary for ligation, mismatches outside the window may also affect productive reactions (52). Reductions in ligation efficiency appear to be more related to reduced primer annealing than to inhibition of the ligase, unless, of course, these polymorphisms mapped to the 4-nt ligation window. Thus, we designed probes to minimize the impact of any mismatches caused by HIV-1 sequence diversity in the region of the targeted drug resistance mutations (see below).

Accommodating HIV-1 variation in oligonucleotide probes. To optimize the oligonucleotide probe for OLA with HIV subtypes A and D, we first sequenced 137 RT-coding sequences from HIV-infected Ugandan mother-infant pairs. The alignment obtained with the ClustalW program (www.ebi.ac.uk/clustalw/) was then analyzed by using MargFreq frequency analysis software (http://sray.med.som.jhmi.edu/SCRoftware/Margfreq/) (see Fig. S1 in the supplemental material). In general, sites harboring infrequent polymorphisms (<10% of the subtype A and D sequences) were treated as nonpolymorphic. Nonstandard bases were embedded in the oligonucleotide at sites which corresponded to highly polymorphic sites in the HIV-1 sequence alignment. Deoxyuracil replaced pyrimidine polymorphisms (C and T), and deoxyinosine was placed at positions with purine polymorphisms (A and G) (Fig. 2). At sites where polymorphisms included transversions, we used deoxyuracil to avoid purine-purine mismatches, which might distort a probe-template duplex to a greater extent than would a pyrimidine-pyrimidine mismatch.

For each SNP analysis, we designed a downstream common oligonucleotide and at least two upstream variable oligonucleotides which abutted each other at the nucleotide of interest. One of these variable oligonucleotides was designed to complement the wild-type genotype, while a second one was designed to complement the drug resistance genotype (Fig. 2). At site 70, two common oligonucleotides (70>C1 and 70>C2) were combined with one of two variable oligonucleotides (70<DW1 and 70<DM1) to detect the wild-type lysine codons (AAA and AAG) and the mutant arginine codons (AGA and AGG). An AAA→AGA change or an AAG→AGG change in the codon codes for arginine at this position. Since both wild-type codons were found in the dominant sequences, we included the third base in the common oligonucleotides at this site such that the upstream site 70 oligonucleotides discriminated between adenosine or guanosine at the second nucleotide in the codon (Fig. 2). At site 103, we designed three variable oligonucleotides, one to detect the wild-type codon (AAA) and one to detect each of the two synonymous drug resistance mutant codons (AAT and AAC). We found no evidence of the lysine synonym AAG at this position. The discriminating base for site 103 was placed in the downstream oligonucleotide such that the upstream oligonucleotide was common for detection of both wild-type and mutant polymorphisms (Fig. 2). The design of the oligonucleotides for site 181 was more straightforward (Fig. 2). The vast majority of only the TGT cysteine codon emerges from the wild-type TAT codon to encode NVP resistance.

Optimization of OLA efficiency. We optimized the OLA reaction for the enzyme, template, and oligonucleotide input concentrations as well as for the salt content and the cycling conditions (Fig. 3). These optimizations were performed by using a clonal template amplified from plasmids containing genes for known HIV RT mutations (K70R, Y181C, and K103N) or wild-type HIV RT genes. The results for the K103N (AAA→AAC) mutation and wild-type sequences are shown in Fig. 3. The use of cloned DNA of known sequence was necessary to control for selectivity and sensitivity. First, the ligase concentration was varied over a 30-fold range, based on the standard concentration previously described for optimal ligation. It is worth mentioning that ligation product formation increased linearly up to 0.5 U/μl, which was the highest concentration tested (Fig. 3A). Second, the oligonucleotide input was optimized (Fig. 3B). In any given reaction, both oligonucleotides are present in equimolar amounts, such that the *x* axis reports the additive quantities of the two oligonucleotides. The amount of the specific reaction product (wild-type K103) was high and relatively insensitive to salt in the range of 10 to 50 mM KCl (Fig. 3C, dashed line). Based on these controls, all subsequent ligation reactions included 0.2 U/μl of ligase in a reaction mixture containing 10 mM KCl and 0.75 pmol/μl each

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A

Oligo Name		Amino Acid
70<DW1	*GCUATAAAGAAAAAAGAUAGUACTAA	Lys (wt)
70<DM1	*GCUATAAAGAAAAAAGAUAGUACTAG	Arg (dr)
70>DC1	ATGGAGIAAAUTAGTIGATTTUAGAGA	
70>DC2	GTGGAGIAAAUTAGTIGATTTUAGAGA	

Patient		Subtype
MTN207	TATTC--G----------C--C-----A-----G---T----A--C--C-----GCTCA	A
MTA077	TATTT--C----------T--C-----A-----A---T----G-----C-----GCTCA	A/D/A
MTU035	TATTT--T----------C--C-----G-----G---T----A-----C-----GCTCA	A/D
MTN079	TATTT--T----------T--C-----A-----A---T----G-----C-----GCTCA	A
INU183	TATTT--A----------G--C--T-----G-----A---T----A-----C-----ACTTA	D
INA175	TATTT--T----------T--C-----A-----A---T----G-----T-----ACTCA	A
MTA071	TATTT--C----------C--T-----G-----A---C----A-----C-----ACTTA	D
MTN211	TATTT--C----------C--T-----G-----A---C----A-----C-----ACTTA	D
INA094	TATTT--C----------C--T-----G-----A---T----A-----T-----ACTTA	D
INA098	TATTT--A----------C--T-----G-----A---T----A-----T-----ACTTA	D
INA173	TATTT--T----------T--C-----A-----A---T----A-----T-----GCTCA	A
MTU176	TATTT--T----------C--T-----A-----A---T----A-----C-----GCTCA	A
MTN208	TATTT--T----------C--T-----G-----A---T----A-----C-----ACTTA	D
INU026	TATTT--T----------T--C-----A-----A---T----A-----T-----GCTCA	A
MTU029	TATTT--T----------C--C-----A-----G---C----A-----C-----GCTCA	A
INU036	TATTT--T----------C--C-----G-----G---T----A-----C-----GCTCA	A/D

B

Oligo Name	Site	Oligo Sequence: Upstream (5' radiolabeled)	Oligo Sequence: Downstream	Amino Acid Specified
103<C1		CATCCUGCAGGUUTAAAAAAGAA		
103>DW1	103		AAAATCAGTAACAGTACTIGATGTGG	Lys (wt)
103>DM1	103		TAAATCAGTAACAGTACTIG	Asn (dr)
103>DM2	103		CAAATCAGTAACAGTACTIG	Asn (dr)
181<DW	181	AAAATCCAGAAATIITTATCTA		Tyr (wt)
181<DM	181	AAAATCCAGAAATIITTATCTG		Cys (dr)
181>C1			TCAATACATGGATGACTTGTATGTAGGA	

FIG. 2. Design and sequence of oligonucleotide probes for OLA directed at codons 70, 103, and 181 in the HIV-1 RT-coding sequence. A frequency analysis for the entire cohort was used to design the oligonucleotides, a full summary of which is provided in the supplementary material. Promiscuous nonstandard bases are underlined. Asterisks denote the position of the 5′ end label. The codons of interest are shaded. The nucleotides which confer amino acid changes are shown in boldface. The amino acids encoded by each codon are shown at the right (A). Probes for site 70 are shown aligned to representative dominant sequences from a MTCT cohort. (B) Probes for sites 70, 103 and 181 are shown. wt, wild type; dr, drug resistant. The nomenclature for the probes is as follows: the first number indicates the site; < denotes upstream; > denotes downstream; D and C denote whether an oligonucleotide is discriminating or common, respectively; and W and M denote wild type and mutant, respectively. We have arbitrarily assigned a "1" or a "2" to oligonucleotides which probe for synonymous codons.

of the two oligonucleotides. When low levels of NVP resistance mutations in an HIV-1 quasispecies were examined, it was necessary to use a maximal template amount (25 ng or 2 ng/μl) for the detection of the mutation. However, the use of the wild-type oligonucleotides with 25 ng PCR product from a patient sample typically resulted in a level of ligation beyond the linear range of the assay. Thus, we also performed ligations with 5 ng (0.4 ng/μl) of template with both the wild-type and the mutant oligonucleotide pairs.

Ligation cycles were increased to optimize the amount of ligation product and the detection of low-frequency SNPs in a template population (Fig. 3D and E). DNA template from HIV-1 wild-type and K103N clones were mixed to achieve 50%, 10%, 3%, 1%, and 0% mutant contents, such that each mixture contained 5 ng/μl total DNA. The cycle number was optimized to obtain increased sensitivity with low percentages of mutant contents (Fig. 3D and E). At 20 and 30 reaction cycles, OLA could easily detect 1 and 3% mutant contents, but the amount of the ligation product reached a plateau between the 10% and 50% mutant frequencies. For example, if a patient harbors an HIV-1 population with 1% K103N, 25 ng of template would saturate the level of ligation with the wild-type probes, whereas optimal ligation would be obtained with the mutant K103N probes. However, ligation of wild-type probes would be in the linear range with 5 ng of template but the 1% mutant content would not be detected with 5 ng of template.

OLA specificity and universality for diverse HIV-1 sequences. The specificities and ligation efficiencies were examined for all of the wild-type and mutant oligonucleotide pairs at sites 70, 103, and 181. Figure 4 summarizes the results for the specificity and sensitivity of the 103 probes for ligation with known subtype A templates of increasing concentration (0.25 to 50 ng). The clone MTN207-6 template harbored a wild-type codon at site 103 (AAA), whereas the mutant Asn codon (AAC) and Asn (AA-T) were found in clones MTN207-4 and MTN148-28, respectively (all clones were subtype A). In general, higher levels of specific ligated oligonucleotides were recovered with increasing concentrations of template (from 0.25 to 18 ng). The ligation reaction was inhibited with amounts of template above 25 ng, suggesting that the excess DNA template was trapping the enzyme (Fig. 4). A very low level of background ligation was observed with the mutant AA-T and AA-C oligonucleotide sets on the (AAA) MTN207-6 wild-type template compared to the level of background ligation observed with specific wild-type oligonucleotide set (Fig. 4A and B). A similar specificity was found with the site 103-specific

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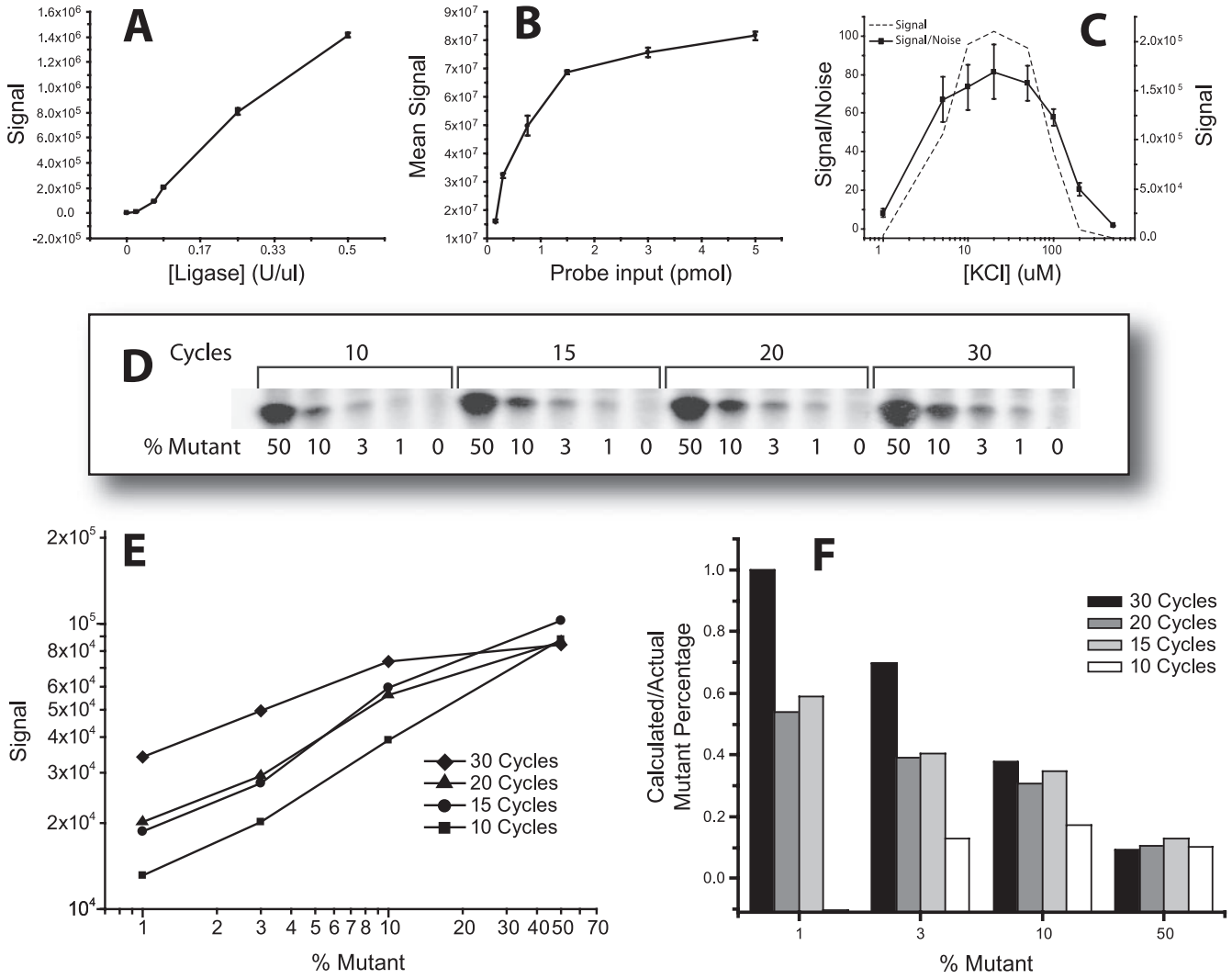


FIG. 3. (A) Ligation product as a function of ligase input; (B) ligation product as a function of probe input. Both probes were present in equimolar amounts (*x* axis). For example, the right-most datum point was the result of oligonucleotide ligation when both oligonucleotide 103<C1 and oligonucleotide 103>DW1 were present at 2.5 pmol. (C) The dotted line shows the relative ligation product from the wild-type site 103 oligonucleotides on a wild-type template. The solid line is the ratio of the correct ligation signal to the incorrect ligation signal (i.e., mutant 103>DM2) on a wild-type template. The data in panels A to C were obtained by using 0.75 pmol 5′ ^{32}P-labeled 103<C1 oligonucleotide, 0.75 pmol 103>DW1 oligonucleotide, 2.5 U Ampligase DNA ligase, 10 mM KCl, and 25 ng template MTN207-6, except where noted otherwise. (D) Autoradiograph showing 25 ng of five template mixtures containing 50%, 10%, 3%, 1%, and 0% mutant template subjected to increasing OLA thermal cycling. (E) Traces of the data shown in panel D. (F) Densitometry values were used to calculate the signal per 1% mutant content. Bars represent the signal given by each percentage in all four mixtures with all cycling conditions as a fraction of that given by the 1% mutant mixture at 30 OLA cycles.

probe sets with a MTN207-4 (site 103 AAC) mutant template. However, significant background ligation was observed with the site 103 AA-C oligonucleotide set and the MTN148-28 (site 103 AAT) template (Fig. 4C). This background ligation with the AAC mutant probe set on a mutant template is of little consequence when the fraction of mutant N103 compared with the fraction of wild-type K103 in the HIV-1 population of a patient is reported.

To test the universality of the mutant and wild-type probe sets, we probed a genetically diverse panel of HIV-1 clones with all probes for sites 70, 103, and 181 (Fig. 5). These clones were derived from drug-naïve or ARV-treated Ugandan patients infected with either subtype A or subtype D HIV-1 (see Materials and Methods). All clones were sequenced to determine both the codon identities at positions 70, 103, and 181 and the sequence diversity at the oligonucleotide binding sites (Fig. 5; see Fig. S1 in the supplemental material). Overall, we observed a very robust ligation signal from both subtype A and subtype D clones with the correct oligonucleotide probes (Fig. 5). The sequence diversity at specific sites within the target sequence of the oligonucleotides was accommodated with nonstandard bases that maintained base pairing or simply a double-stranded DNA conformation (Fig. 2A; Fig. 5). In addition to these defined polymorphic sites, a few nucleotide substitutions were found scattered in the oligonucleotide binding site, but the frequencies of these mutations generally fell below our 10% cutoff for the inclu-

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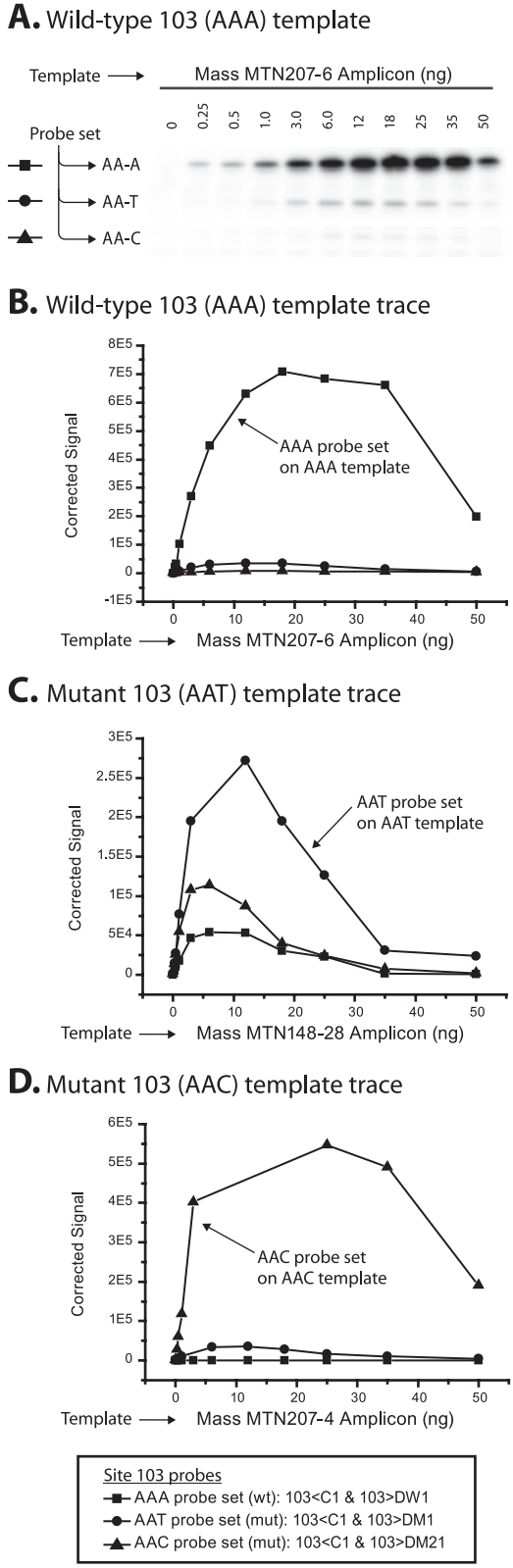


FIG. 4. (A) Autoradiographs showing the ligation products from the correct probe pair 103<C1 and 103>DW1 (AA-A) on the wild-type template as well as ligation products of the mutant-specific probes 103>DM1 (AA-T) and 103>DM2 (AA-C) on the same template; (B) densitometry traces of the autoradiograph shown in panel A; (C) densitometry traces of the ligation products obtained by using the correct probe pair 103<C1 with 103>DM1 (AA-T; circles), the incorrect probe pair 103<C1 with 103>DM2 (AA-C; triangles), and the incorrect probe pair 103<C1 with 103>DW1 (AAA; squares); (D) densitometry traces of the ligation products obtained by using the correct probe pair 103<C1 with 103>DM2 (AA-C; triangles), the incorrect probe pair 103<C1 with 103>DM1 (AA-T; circles), and the incorrect probe pair 103<C1 with 103>DW1 (AAA; squares). wt, wild type; mut, mutant.

sion of a nonstandard base (see Fig. S1 in the supplemental material). This low frequency of heterogeneity, with some exceptions (INU92-1, site 103), did not appear to affect the ligation efficiency, suggesting that the DNA ligase was not affected by minor sequence mismatches outside of the 4-nt recognition window (Fig. 5B). With most clones, we observed very low background ligation, e.g., ligation reactions on a mutant template with probes specific for the wild-type codon.

The greatest level of background ligation was observed when the codon at amino acid position 70 in RT was probed (Fig. 5A). With the exception of MTA308-4 and MTA341-1, the oligonucleotides used to detect the AAG and AAA wild-type codons or the AGG and AGA mutant codons showed significant cross-reactivity. These findings suggest that the weak G-T base pairing could contribute to this higher level of background ligation. In the case of position 70, the cross-reactivity of these probes has no impact on detection of the K70R mutation in the HIV-1 quasispecies, since the frequency of mutated Arg to that of wild-type Lys would be calculated from the sum of the number of ligation products obtained with mutant probes (AGA + AAA) divided by the number of ligation products obtained with all probe sets (both the wild-type and the mutant probe sets). However, weak G-T base pairing at the discriminatory nucleotide could result in high background levels and a lower sensitivity by the OLA.

Aside from the cross-reactivity caused by G-to-T base pairing, we have found a higher degree of fidelity for base pairing 5′ to the ligation site (the 3′ end of the upstream oligonucleotide) than for base pairing 3′ to the ligation site. Site 181 oligonucleotides (5′ discrimination) were more specific and gave less nonspecific ligation, whereas site 70 oligonucleotides (3′ discrimination) were more cross-reactive (Fig. 4C; Fig. 5A and B). As described above, the reduced fidelity at site 70 did not present a problem, since cross-reactivity was limited to the wild-type or the mutant synonymous codons. Considering these differential fidelities, we suspect that Ampligase is more selective for correct base pairing at the 5′ position of the upstream oligonucleotide than at the 3′ position of the downstream probe (compare Fig. 5B and C). This asymmetric fidelity might aid in the design of oligonucleotides that can be used to probe for other SNPs.

In addition to asymmetric fidelity in the ligase, it is not clear how upstream mismatches affect ligation efficiency or fidelity. In Fig. 5A, clones MTA308-3 and MTN121-1 (AAG at site 70) directed nonspecific ligation of the AAA probe set. Ligation must then have occurred, despite an A-C mismatch downstream of the ligation-competent site. MTA308-4 and MTA341-1 did not exhibit the same nonspecific ligation of the AGA probe set at their AGG codons (a similar A-C mismatch). Although the G→A polymorphism at the second base

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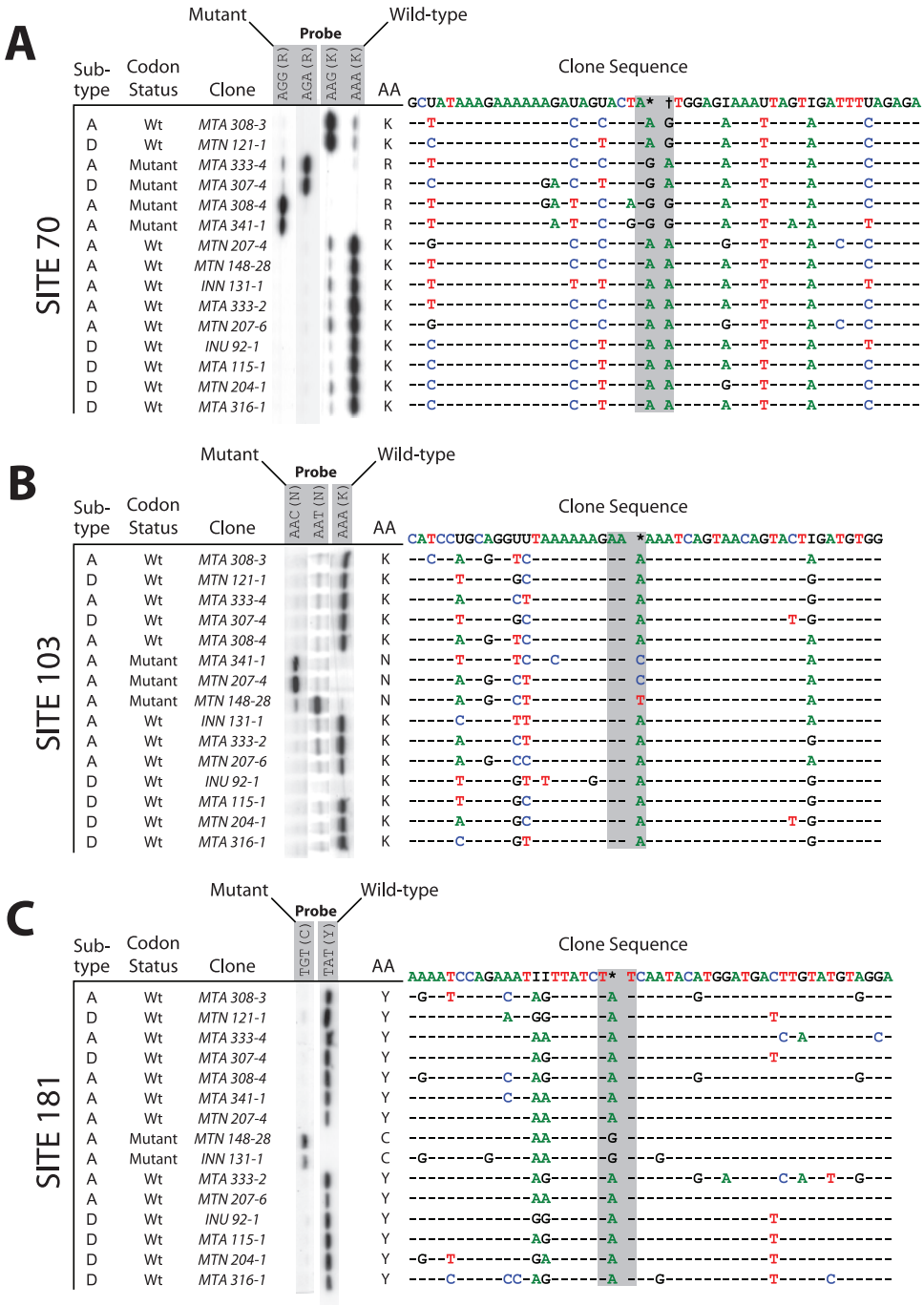


FIG. 5. OLA of a diverse panel of clones with mutant and wild-type probes for sites 70, 103, and 181. The oligonucleotide nomenclature and the general specificity of the probes are shown opposite each clonal template. The sequences of the clone at each drug resistance site is shown at the right. The codons of interest are shown in gray boxes. The general probe sequence is the first sequence for each site. The diverse clone panel was probed with site 70 oligonucleotides (A), site 103 oligonucleotides (B), and site 181 oligonucleotides (C).

in codon 70 may offer an explanation, this is probably not the case, as clones MTA333-4 and MTA307-4 (both with AGA at site 70) directed nonspecific ligation of the AGG probe set (Fig. 5A). The higher fidelity obtained with MTA308-4 and MTA341-1 might then be attributed to a pyrimidine-pyrimidine (T-T and T-C in MTA308-4 and MTA341-1, respectively) mismatch just upstream of the ligase window, a condition which would have been expected to ablate ligation completely. At site 103 (Fig. 5B), clone INU92-1 failed to direct even specific ligation, presumably because of an upstream purine-pyrimidine (A-T) mismatch.

Assessing mutation frequencies in clonal mixtures. When HIV-infected patient samples are analyzed, SNPs such as drug resistance mutations may be found in a small fraction of the

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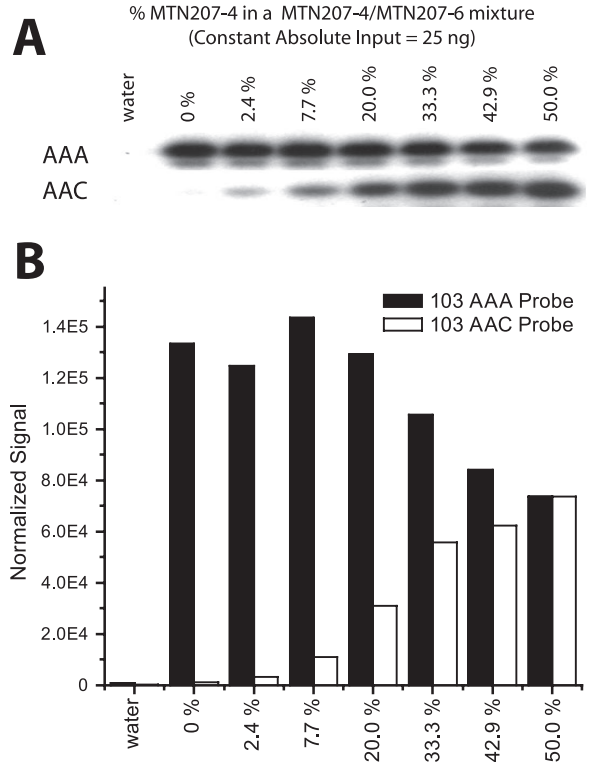


FIG. 6. (A) OLA was performed with probes 103<C1 and 103>DW1 (AAA; top row) and 103<C1 and 103>DM2 (AAC; bottom row) on 25 ng of several mixtures of MTN207-6 and MTN 207-4 templates; (B) intensities of the bands in panel A as a function of the MTN207-4 content. The data were normalized to the 103<C1 ligation to 103>DM2 by use of a 1:1 mixture.

HIV-1 clones found in quasispecies (34, 43). However, even a small percentage of drug-resistant clones could have an impact on the treatment outcome (27). Thus, it was important to determine the level of OLA sensitivity in controlled mixtures of site 103 mutant and wild-type clones. The MTN207-4 mutant clone was mixed with the MTN207-6 wild-type clone such that mutant content was 50%, 42.9%, 33%, 20%, 7.7%, or 2.4% of the wild-type content. A total of 25 ng of this template mixture was then used in triplicate ligation reactions specific for wild-type and mutant codons AAA and AAC, respectively (Fig. 6A). The densitometry data plotted in Fig. 6B show a mutant content-dependent increase in the signal from the AAC probe and a wild-type-dependent decrease in the signal from the AAA probe. The same experimental protocol was performed for site 70 and site 181 mutant detection versus wild-type detection in a mixture, and similar results were obtained (data not shown).

Determining clinical utility of modified OLA. This modified, radioactive OLA was tested for its sensitivity and accuracy in determining the frequency of drug resistance mutations in a patient's quasispecies (Table 1). Briefly, the HIV-1 RT region was amplified by PCR from PBMC samples of HIV-infected mothers treated with NVP. The amount of ligation products from the PCR-amplified patient samples was compared to the amounts on a standard curve generated from the OLA ligation products of known template copy numbers. The percentage of mutant versus the percentage of wild type was then calculated as described in the Materials and Methods. For all four patient samples, OLAs were performed in quintuplicate. A very small variance (Table 1) in the estimated mean mutation frequency was observed. To confirm that OLA was accurately estimating the mutant content, the PCR products used for OLA were also cloned and sequenced to determine the actual mutation frequency, as described in Materials and Methods. The sequencing results were in complete agreement with the OLA results in these analyses. Patient sample MTN228 had an estimated OLA K103N frequency of 3.8% ± 0.5%, whereas 8 of 200 MTN228 clones (1.5%) had the K103N mutation. Similar results were observed with MTN148, MTN207, and MTN218 (Table 1). It is important to note that calculation of the mutation frequency by sequencing analyses of clones is not absolute and can be biased due to sampling error.

Determining clinical cutoffs for OLA with plasma and PBMC samples. We used approximately 5 ng of extracted PBMC DNA (or 5 μl from 100 μl DNA extracted from 10^6 PBMCs) in the PCR amplification. However, the actual copy number of the proviral genomes in this sample could have been insufficient for the analysis of low-frequency SNPs. In this case, oversampling during PCR amplification could provide inaccurate estimates of the mutation frequency or high rates of false-negative results for low-frequency SNPs. In other words, an HIV-1 DNA copy number of 139,000/ml (Table 2, patient sample MTN224) implies that a total of 700 copies were present in the 5 μl added to the PCR amplification mixture and were then probed by OLA. Thus, a 0.14% mutant content can theoretically be detected by OLA. As described in Table 2, the DNA copy numbers ranged from <2,000 to 139,000 in PBMC extracts of 17 patient samples.

With the exception of two samples, the detection of all estimated percentages of mutant versus wild-type frequencies at sites 103 and 181 was theoretically possible, based on HIV-1 DNA load measurements. Sample MTN206 had a 1.2% K103N mutant frequency (Table 2), but according to TaqMan

TABLE 1. Comparison of repetitive OLA analyses with clone frequency

Patient sample	Bulk OLA result (% with 103N)							Clonal analysis	
	Test 1	Test 2	Test 3	Test 4	Test 5	Mean	SD	No. of clones tested	% with 103N
MTN148	98.3	98.2	98.8	98.6	98.7	98.5	0.2	13	100
MTN207	40.3	47.4	44.8	45.5	44.9	44.6	26	8	62.5
MTN218	52.6	39.4	29.6	52.8	36.5	42.2	10.2	15	33.3
MTN228	4.0	3.7	4.6	3.2	3.7	3.8	0.5	200	1.5

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TABLE 2. Comparison of drug resistance found in PBMC HIV-1 DNA to drug resistance found in plasma RNA and pool size of each

Patient sample	PBMCs			Plasma	
	No. of DNA copies/ml	% with K103N	% with Y181C	No. of RNA copies/ml	% with Y181C
MTN203	5,752	<0.4	<0.4	30,817	<0.4
MTN204	9,124	<0.4	<0.4	75,673	<0.4
MTN206	<2,000	1.2	<0.4	345,923	<0.4
MTN207	7,219	44.6	<0.4	NA[a]	<0.4
MTN208	22,487	1.3	63.5	131,370	<0.4
MTN209	7,072	2.0	5.6	132,515	83.9
MTN210	5,406	2.2	92.5	121,239	<0.4
MTN211	5,481	<0.4	<0.4	470,120	<0.4
MTN213	17,310	0.6	<0.4	87,383	<0.4
MTN215	<2,000	<0.4	<0.4	37,075	<0.4
MTN216	5,673	<0.4	<0.4	152,473	<0.4
MTN217	<2,000	<0.4	<0.4	823,356	<0.4
MTN223	33,296	<0.4	<0.4	263,651	<0.4
MTN224	139,453	0.6	0.8	325,935	<0.4
MTN225	6,647	0.5	<0.4	43,500	<0.4
MTN226	3,408	1.1	<0.4	29,787	<0.4
MTN228	<2,000	3.8	3.8	12,707	<0.4

[a] NA, viral load data were not available for patient MTN207.

DNA PCR calculations, less than 10 HIV-1 genomes were sampled from this patient. In this case, it appears that the OLA results are in error, considering that a mutant percentage of only >10% is possible. MTN228 had an estimated 3.8% mutant content at both sites 103 and 108, but less than 10 virus genomes were sampled (Table 2). However, the typical standard deviation for viral RNA or DNA load measurement is at least 0.5 log of the mean. An underestimate of the viral load is likely for sample MTN228, considering that a 3.7 to 4.6% K103N frequency was consistently observed over multiple OLA replicates. In addition, the mutant K103N frequency was estimated to be 1.5% by clonal sequencing (Table 1).

The OLA results for the Y181C mutation derived from PBMC DNA were compared to those derived from plasma RNA (which was reverse transcribed to cDNA). The Y181C mutation was detected in only one plasma sample, whereas it was detected in five samples of PBMC DNA. Undersampling appears to be a greater issue for plasma samples than for PBMC samples, even though viral loads are typically higher in the latter types of samples. RNA for OLA was obtained by the Roche 1.5 Amplicor procedure. An equivalent of 25 μl of plasma (or 1/40 of the number of viral RNA copies/ml) was used in the OLA, i.e., an amount similar to that used for viral RNA load determination. It is not clear why Y181C was not detected in the plasma RNA samples of MTN208, MTN210, and MTN228, considering that sufficient RNA copies were sampled (Table 2). Unlike the OLA performed with PBMC DNA, the viral RNA from plasma must first be reverse transcribed to cDNA and then amplified by PCR. The reverse transcription step is not cycled, and thus the conversion of RNA to cDNA may be a fairly inefficient process (less than 50% with contaminating nucleic acids) (29). As discussed above, inefficient RNA-to-cDNA conversion may have been responsible for the reduced sensitivity of OLA with the plasma RNA sample. However, it is also possible that the PBMC DNA represents an archive of HIV-1 DNA with NVP resistance mutations that may persist longer than the relatively short-lived HIV-1 particles in plasma.

DISCUSSION

We have developed a highly sensitive and specific assay for the detection of NVP resistance mutations present in a minority of clones within the HIV quasispecies. This assay can detect NVP resistance mutations equally well in both subtype A and D isolates of HIV, the major subtypes present in east-central Africa (Uganda and Kenya). Experiments with multiple NVP-resistant mutant and wild-type clones were performed in order to carefully control for any confounding background ligation. In addition to rigorous controls, the optimal conditions for ligation and low-level SNP detection were established by the use of various concentrations of salt, ligase, template, and oligonucleotides. We also compared the detection of NVP resistance mutations in a patient's HIV-1 quasispecies using OLA and performing clonal sequencing analyses. On the basis of these findings, a radioactivity-based OLA may be the most sensitive, reliable, and controlled system for the detection of low-level drug resistance mutations in HIV-infected patients. In contrast, other assay systems that have been developed, such as allele-specific PCR, were not as rigorously controlled with patient samples or clones and may provide false-positive readings for drug resistance mutations present at levels below 0.1%.

One major difficulty in the application of OLA or other techniques for the detection of SNPs in HIV isolates is the extensive genetic diversity of the HIV-1 genome. Unlike allele-specific PCR, OLA is less sensitive to genetic diversity outside of the narrow 4-nt window of ligase specificity located at the gap between the abutted oligonucleotides. Nonetheless, the diversity of the HIV-1 genome could still affect both the specificity and the sensitivity of OLA when samples from patients in the developed world are analyzed. Our recent studies with OLA have focused on NVP resistance mutations that may emerge in NVP-treated mothers and their infants. These studies are based in Uganda, where subtypes A and D typically cocirculate. To provide an accommodation for the genetic diversity between subtypes A and D, we designed oligonucleotides with nonstandard nucleotides at heterogeneous sites adjacent to the drug resistance mutations. These modified oligonucleotides maintained the base pairing or retained the DNA duplex conformation for the vast majority of HIV-1 isolates in our cohort, but more importantly, they were efficient substrates for specific ligation on the template. In the original protocol, a high degree of sequence variability in the template was typically associated with ligation failure (52), the probable cause of assay error (23). Since both wild-type and mutant oligonucleotides have the same sequence, aside from the discriminating nucleotide, mismatches which disrupt annealing or ligation are problematic. Sequencing analyses of each of the patient samples and the clones used in this study revealed considerable genetic diversity near the mutation sites of interest. In fact, the vast majority of sequence variation near the ligation window was accommodated by the nonstandard bases incorporated into the oligonucleotides.

Although the OLA was highly sensitive and specific, we did observe a low background ligation signal with specific primary

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HIV-1 sequences of subtypes A and D. The low background signal simply decreased the overall sensitivity of mutant detection in a population from approximately 0.01% to approximately 0.4%. As described above, we established this conservative cutoff on the basis of the findings of experiments performed with genetically diverse clinical isolates and with conditions of in vitro mixing of known clones. For example, a low level of background ligation was observed with clones MTN207-6 and MTN207-4 from patient MTN207, which had similar sequences at the oligonucleotide binding sites, aside from the AAA and AAC codons, respectively, at position 103 (Fig. 5B). Although when the data were normalized to the probe-specific activity the level of nonspecific ligation was low (compare Fig. 4B to Fig. 5B for MTN207-6), we observed positive and reproducible background signals from these clones with the site 103 probes. In contrast, our findings for sites 70 and 181 suggest that there is a higher fidelity when we probed for mutations with the discriminating base in the upstream oligonucleotide rather than in the downstream oligonucleotide.

We have established rigorous and accurate background levels for OLA, based on these primary HIV-1 sequence controls, which to date has not been performed with other methods for the detection of HIV-1 mutations present at low frequencies. Using simple mixing experiments with known control templates, we can detect mutations that are present at a level as low as 0.01%, but in most instances, this level of detection is impossible for clinical isolates due to the input of HIV-1 templates into the assay mixture. For the detection of a 0.01% mutant content in an HIV-1 population, 10,000 HIV-1 RNA or DNA copies must be placed into the reaction mixture, whether it is for OLA or allele-specific PCR. Given the efficiency of reverse transcription and PCR and our empirical tests of these limits, it is quite likely that viral loads of >1,000,000 RNA copies/ml or >200,000 DNA copies/ml are required to achieve a 0.01% detection limit. We have established a more conservative SNP limit of 0.4% and found that accurate levels of K103N or Y181C mutations could easily be detected from PBMC DNA but not from plasma viral RNA. These results suggest that the conversion of RNA to cDNA is fairly inefficient, such that higher viral loads are necessary for analyses of SNPs. In addition, it is also quite possible that the K103N and Y181C mutations are archived in the PBMC population but are not found in the circulating virus population. It is still unclear if the archived or circulating population of virus will have a greater effect on the subsequent treatment outcome. These studies are still under way.

While K103N and Y181C are certainly important NVP resistance mutations, other mutations have been associated with NVP resistance after the administration of a single dose, such as Y188C (26). Following the standardization of OLA with radiolabeled oligonucleotides, we are now using a downstream fluorophore-labeled oligonucleotide and an upstream oligonucleotide with a short "zip code" tag sequence. This zip code sequence anneals to one of a hundred different fluorescent beads, which are then analyzed by three-color flow fluorimetry. This new, high-throughput technology will permit us to probe for multiple HIV-1 drug resistance mutations and wild-type sequences in a single reaction.

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REFERENCES

1. **Arien, K. K., G. Vanham, and E. J. Arts.** 2007. Is HIV-1 evolving to a less virulent form in humans? Nat. Rev. Microbiol. **5:**141–151.
2. **Bajunirwe, F., I. Massaquoi, S. Asiimwe, M. R. Kamya, E. J. Arts, and C. C. Whalen.** 2004. Effectiveness of nevirapine and zidovudine in a pilot program for the prevention of mother-to-child transmission of HIV-1 in Uganda. Afr. Health Sci. **4:**146–154.
3. **Beck, I. A., M. Mahalanabis, G. Pepper, A. Wright, S. Hamilton, E. Langston, and L. M. Frenkel.** 2002. Rapid and sensitive oligonucleotide ligation assay for detection of mutations in human immunodeficiency virus type 1 associated with high-level resistance to protease inhibitors. J. Clin. Microbiol. **40:**1413–1419.
4. **Brenner, B. G., and M. A. Wainberg.** 2000. The role of antiretrovirals and drug resistance in vertical transmission of HIV-1 infection. Ann. N. Y. Acad. Sci. **918:**9–15.
5. **Casado, J. L., K. Hertogs, L. Ruiz, F. Dronda, A. Van Cauwenberge, A. Arno, I. Garcia-Arata, S. Bloor, A. Bonjoch, J. Blazquez, B. Clotet, and B. Larder.** 2000. Non-nucleoside reverse transcriptase inhibitor resistance among patients failing a nevirapine plus protease inhibitor-containing regimen. AIDS **14:**F1–F7.
6. **Collins, J. A., M. G. Thompson, E. Paintsil, M. Ricketts, J. Gedzior, and L. Alexander.** 2004. Competitive fitness of nevirapine-resistant human immunodeficiency virus type 1 mutants. J. Virol. **78:**603–611.
7. **Connor, E. M., R. S. Sperling, R. Gelber, P. Kiselev, G. Scott, M. J. O'Sullivan, R. VanDyke, M. Bey, W. Shearer, R. L. Jacobson, et al.** 1994. Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. N. Engl. J. Med. **331:**1173–1180.
8. **Coovadia, H.** 2004. Antiretroviral agents—how best to protect infants from HIV and save their mothers from AIDS. N. Engl. J. Med. **351:**289–292.
9. **Edelstein, R. E., D. A. Nickerson, V. O. Tobe, L. A. Manns-Arcuino, and L. M. Frenkel.** 1998. Oligonucleotide ligation assay for detecting mutations in the human immunodeficiency virus type 1 *pol* gene that are associated with resistance to zidovudine, didanosine, and lamivudine. J. Clin. Microbiol. **36:**569–572.
10. **Ellis, G. M., M. Mahalanabis, I. A. Beck, G. Pepper, A. Wright, S. Hamilton, S. Holte, W. E. Naugler, D. M. Pawluk, C. C. Li, and L. M. Frenkel.** 2004. Comparison of oligonucleotide ligation assay and consensus sequencing for detection of drug-resistant mutants of human immunodeficiency virus type 1 in peripheral blood mononuclear cells and plasma. J. Clin. Microbiol. **42:**3670–3674.
11. **Eshleman, S. H., L. A. Guay, A. Mwatha, E. R. Brown, S. P. Cunningham, P. Musoke, F. Mmiro, and J. B. Jackson.** 2004. Characterization of nevirapine resistance mutations in women with subtype A vs. D HIV-1 6–8 weeks after single-dose nevirapine (HIVNET 012). J. Acquir. Immune. Defic. Syndr. **35:**126–130.
12. **Eshleman, S. H., L. A. Guay, A. Mwatha, S. P. Cunningham, E. R. Brown, P. Musoke, F. Mmiro, and J. B. Jackson.** 2004. Comparison of nevirapine (NVP) resistance in Ugandan women 7 days vs. 6–8 weeks after single-dose NVP prophylaxis: HIVNET 012. AIDS Res. Hum. Retrovir. **20:**595–599.
13. **Eshleman, S. H., and J. B. Jackson.** 2002. Nevirapine resistance after single dose prophylaxis. AIDS Rev. **4:**59–63.
14. **Eshleman, S. H., D. Jones, J. Galovich, E. E. Paxinos, C. J. Petropoulos, J. B. Jackson, and N. Parkin.** 2006. Phenotypic drug resistance patterns in subtype A HIV-1 clones with nonnucleoside reverse transcriptase resistance mutations. AIDS Res. Hum. Retrovir. **22:**289–293.
15. **Eshleman, S. H., P. Krogstad, J. B. Jackson, Y. G. Wang, S. Lee, L. J. Wei, S. Cunningham, M. Wantman, A. Wiznia, G. Johnson, S. Nachman, and P. Palumbo.** 2001. Analysis of human immunodeficiency virus type 1 drug resistance in children receiving nucleoside analogue reverse-transcriptase inhibitors plus nevirapine, nelfinavir, or ritonavir (Pediatric AIDS Clinical Trials Group 377). J. Infect. Dis. **183:**1732–1738.
16. **Eshleman, S. H., M. Mracna, L. A. Guay, M. Deseyve, S. Cunningham, M. Mirochnick, P. Musoke, T. Fleming, F. M. Glenn, L. M. Mofenson, F. Mmiro, and J. B. Jackson.** 2001. Selection and fading of resistance mutations in women and infants receiving nevirapine to prevent HIV-1 vertical transmission (HIVNET 012). AIDS **15:**1951–1957.
17. **Esnouf, R., J. Ren, C. Ross, Y. Jones, D. Stammers, and D. Stuart.** 1995. Mechanism of inhibition of HIV-1 reverse transcriptase by non-nucleoside inhibitors. Nat. Struct. Biol. **2:**303–308.
18. **Flys, T., D. V. Nissley, C. W. Claasen, D. Jones, C. Shi, L. A. Guay, P. Musoke, F. Mmiro, J. N. Strathern, J. B. Jackson, J. R. Eshleman, and S. H. Eshleman.** 2005. Sensitive drug-resistance assays reveal long-term persistence of HIV-1 variants with the K103N nevirapine (NVP) resistance mu-

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tation in some women and infants after the administration of single-dose NVP: HIVNET 012. J. Infect. Dis. **192:**24–29.
19. **Frenkel, L. M., L. E. Wagner, S. M. Atwood, T. J. Cummins, and S. Dewhurst.** 1995. Specific, sensitive, and rapid assay for human immunodeficiency virus type 1 *pol* mutations associated with resistance to zidovudine and didanosine. J. Clin. Microbiol. **33:**342–347.
20. **Gale, H. B., V. L. Kan, and R. C. Shinol.** 2006. Performance of the TruGene human immunodeficiency virus type 1 genotyping kit and OpenGene DNA sequencing system on clinical samples diluted to approximately 100 copies per milliliter. Clin. Vaccine Immunol. **13:**235–238.
21. **Garcia-Bujalance, S., C. L. de Guevara, J. Gonzalez-Garcia, J. R. Arribas, and A. Gutierrez.** 2005. Comparison between sequence analysis and a line probe assay for testing genotypic resistance of human immunodeficiency virus type 1 to antiretroviral drugs. J. Clin. Microbiol. **43:**4186–4188.
22. **Guay, L. A., P. Musoke, T. Fleming, D. Bagenda, M. Allen, C. Nakabiito, J. Sherman, P. Bakaki, C. Ducar, M. Deseyve, L. Emel, M. Mirochnick, M. G. Fowler, L. Mofenson, P. Miotti, K. Dransfield, D. Bray, F. Mmiro, and J. B. Jackson.** 1999. Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: HIVNET 012 randomised trial. Lancet **354:**795–802.
23. **Halvas, E. K., G. M. Aldrovandi, P. Balfe, I. A. Beck, V. F. Boltz, J. M. Coffin, L. M. Frenkel, J. D. Hazelwood, V. A. Johnson, M. Kearney, A. Kovacs, D. R. Kuritzkes, K. J. Metzner, D. V. Nissley, M. Nowicki, S. Palmer, R. Ziermann, R. Y. Zhao, C. L. Jennings, J. Bremer, D. Brambilla, and J. W. Mellors.** 2006. Blinded, multicenter comparison of methods to detect a drug-resistant mutant of human immunodeficiency virus type 1 at low frequency. J. Clin. Microbiol. **44:**2612–2614.
24. **Iglesias-Ussel, M. D., C. Casado, E. Yuste, I. Olivares, and C. Lopez-Galindez.** 2002. In vitro analysis of human immunodeficiency virus type 1 resistance to nevirapine and fitness determination of resistant variants. J. Gen. Virol. **83:**93–101.
25. **Jackson, J. B., G. Becker-Pergola, L. A. Guay, P. Musoke, M. Mracna, M. G. Fowler, L. M. Mofenson, M. Mirochnick, F. Mmiro, and S. H. Eshleman.** 2000. Identification of the K103N resistance mutation in Ugandan women receiving nevirapine to prevent HIV-1 vertical transmission. AIDS **14:**F111–F115.
26. **Johnson, J. A., J. F. Li, L. Morris, N. Martinson, G. Gray, J. McIntyre, and W. Heneine.** 2005. Emergence of drug-resistant HIV-1 after intrapartum administration of single-dose nevirapine is substantially underestimated. J. Infect. Dis. **192:**16–23.
27. **Jourdain, G., N. Ngo-Giang-Huong, C. S. Le, C. Bowonwatanuwong, P. Kantipong, P. Leechanachai, S. Ariyadej, P. Leenasirimakul, S. Hammer, and M. Lallemant.** 2004. Intrapartum exposure to nevirapine and subsequent maternal responses to nevirapine-based antiretroviral therapy. N. Engl. J. Med. **351:**229–240.
28. **Kapoor, A., M. Jones, R. W. Shafer, S. Y. Rhee, P. Kazanjian, and E. L. Delwart.** 2004. Sequencing-based detection of low-frequency human immunodeficiency virus type 1 drug-resistant mutants by an RNA/DNA heteroduplex generator-tracking assay. J. Virol. **78:**7112–7123.
29. **Kotewicz, M. L., J. M. D'Alessio, K. M. Driftmier, K. P. Blodgett, and G. F. Gerard.** 1985. Cloning and overexpression of Moloney murine leukemia virus reverse transcriptase in Escherichia coli. Gene **35:**249–258.
30. **Lecossier, D., N. S. Shulman, L. Morand-Joubert, R. W. Shafer, V. Joly, A. R. Zolopa, F. Clavel, and A. J. Hance.** 2005. Detection of minority populations of HIV-1 expressing the K103N resistance mutation in patients failing nevirapine. J. Acquir. Immune Defic. Syndr. **38:**37–42.
31. **Margulies, M., M. Egholm, W. E. Altman, S. Attiya, J. S. Bader, L. A. Bemben, J. Berka, M. S. Braverman, Y. J. Chen, Z. Chen, S. B. Dewell, L. Du, J. M. Fierro, X. V. Gomes, B. C. Godwin, W. He, S. Helgesen, C. H. Ho, G. P. Irzyk, S. C. Jando, M. L. Alenquer, T. P. Jarvie, K. B. Jirage, J. B. Kim, J. R. Knight, J. R. Lanza, J. H. Leamon, S. M. Lefkowitz, M. Lei, J. Li, K. L. Lohman, H. Lu, V. B. Makhijani, K. E. McDade, M. P. McKenna, E. W. Myers, E. Nickerson, J. R. Nobile, R. Plant, B. P. Puc, M. T. Ronan, G. T. Roth, G. J. Sarkis, J. F. Simons, J. W. Simpson, M. Srinivasan, K. R. Tartaro, A. Tomasz, K. A. Vogt, G. A. Volkmer, S. H. Wang, Y. Wang, M. P. Weiner, P. Yu, R. F. Begley, and J. M. Rothberg.** 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature **437:**376–380.
32. **Marseille, E., J. G. Kahn, F. Mmiro, L. Guay, P. Musoke, M. G. Fowler, and J. B. Jackson.** 1999. Cost effectiveness of single-dose nevirapine regimen for mothers and babies to decrease vertical HIV-1 transmission in sub-Saharan Africa. Lancet **354:**803–809.
33. **Martinson, N. A., L. Morris, G. Gray, D. Moodley, V. Pillay, S. Cohen, P. Dhlamini, A. Puren, S. Bhayroo, J. Steyn, and J. A. McIntyre.** 2007. Selection and persistence of viral resistance in HIV-infected children after exposure to single-dose nevirapine. J. Acquir. Immune. Defic. Syndr. **44:**148–153.
34. **Mayers, D. L., F. E. McCutchan, E. E. Sanders-Buell, L. I. Merritt, S. Dilworth, A. K. Fowler, C. A. Marks, N. M. Ruiz, D. D. Richman, and C. R. Roberts.** 1992. Characterization of HIV isolates arising after prolonged zidovudine therapy. J. Acquir. Immune Defic. Syndr. **5:**749–759.
35. **Musoke, P., L. A. Guay, D. Bagenda, M. Mirochnick, C. Nakabiito, T. Fleming, T. Elliott, S. Horton, K. Dransfield, J. W. Pav, A. Murarka, M. Allen, M. G. Fowler, L. Mofenson, D. Hom, F. Mmiro, and J. B. Jackson.** 1999. A phase I/II study of the safety and pharmacokinetics of nevirapine in HIV-1-infected pregnant Ugandan women and their neonates (HIVNET 006). AIDS **13:**479–486.
36. **Mwanyumba, F., P. Gaillard, I. Inion, C. Verhofstede, P. Claeys, V. Chohan, S. Vansteelandt, K. Mandaliya, M. Praet, and M. Temmerman.** 2002. Placental inflammation and perinatal transmission of HIV-1. J. Acquir. Immune Defic. Syndr. **29:**262–269.
37. **Mwapasa, V., S. J. Rogerson, J. J. Kwiek, P. E. Wilson, D. Milner, M. E. Molyneux, D. D. Kamwendo, E. Tadesse, E. Chaluluka, and S. R. Meshnick.** 2006. Maternal syphilis infection is associated with increased risk of mother-to-child transmission of HIV in Malawi. AIDS **20:**1869–1877.
38. **Odaibo, G. N., D. O. Olaleye, L. Heyndrickx, K. Vereecken, K. Houwer, and W. Jassens.** 2006. Mother-to-child transmission of different HIV-1 subtypes among ARV naive infected pregnant women in Nigeria. Rev. Inst. Med. Trop. Sao Paulo **48:**77–80.
39. **Petropoulos, C. J., N. T. Parkin, K. L. Limoli, Y. S. Lie, T. Wrin, W. Huang, H. Tian, D. Smith, G. A. Winslow, D. J. Capon, and J. M. Whitcomb.** 2000. A novel phenotypic drug susceptibility assay for human immunodeficiency virus type 1. Antimicrob. Agents Chemother. **44:**920–928.
40. **Quan, Y., B. G. Brenner, R. G. Marlink, M. Essex, T. Kurimura, and M. A. Wainberg.** 2003. Drug resistance profiles of recombinant reverse transcriptases from human immunodeficiency virus type 1 subtypes A/E, B, and C. AIDS Res. Hum. Retrovir. **19:**743–753.
41. **Quinones-Mateu, M. E., and E. J. Arts.** 2002. Fitness of drug resistant HIV-1: methodology and clinical implications. Drug Resist. Update **5:**224–233.
42. **Quinones-Mateu, M. E., S. C. Ball, A. J. Marozsan, V. S. Torre, J. L. Albright, G. Vanham, G. van der Groen, R. L. Colebunders, and E. J. Arts.** 2000. A dual infection/competition assay shows a correlation between ex vivo human immunodeficiency virus type 1 fitness and disease progression. J. Virol. **74:**9222–9233.
43. **Richman, D. D., D. Havlir, J. Corbeil, D. Looney, C. Ignacio, S. A. Spector, J. Sullivan, S. Cheeseman, K. Barringer, and D. Pauletti.** 1994. Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. J. Virol. **68:**1660–1666.
44. **Schuurman, R., D. Brambilla, T. de Groot, D. Huang, S. Land, J. Bremer, I. Benders, and C. A. Boucher.** 2002. Underestimation of HIV type 1 drug resistance mutations: results from the ENVA-2 genotyping proficiency program. AIDS Res. Hum. Retrovir. **18:**243–248.
45. **Shackelford, W., S. Deng, K. Murayama, and J. Wang.** 2004. A new technology for mutation detection. Ann. N. Y. Acad. Sci. **1022:**257–262.
46. **Shi, C., S. H. Eshleman, D. Jones, N. Fukushima, L. Hua, A. R. Parker, C. J. Yeo, R. H. Hruban, M. G. Goggins, and J. R. Eshleman.** 2004. LigAmp for sensitive detection of single-nucleotide differences. Nat. Methods **1:**141–147.
47. **Taylor, D. L., P. S. Ahmed, P. Chambers, A. S. Tyms, J. Bedard, J. Duchaine, G. Falardeau, J. F. Lavallee, W. Brown, R. F. Rando, and T. Bowlin.** 1999. Pyrido [1,2a] indole derivatives identified as novel non-nucleoside reverse transcriptase inhibitors of human immunodeficiency virus type 1. Antivir. Chem. Chemother. **10:**79–86.
48. **Tsongalis, G. J., T. Gleeson, M. Rodina, D. Anamani, J. Ross, I. Joanisse, L. Tanimoto, and R. Ziermann.** 2005. Comparative performance evaluation of the HIV-1 LiPA protease and reverse transcriptase resistance assay on clinical isolates. J. Clin. Virol. **34:**268–271.
49. **Vermund, S. H.** 2004. Prevention of mother-to-child transmission of HIV in Africa. Top. HIV Med. **12:**130–134.
50. **Villahermosa, M. L., I. Beck, L. Perez-Alvarez, G. Contreras, L. M. Frenkel, S. Osmanov, E. V. de Parga, E. Delgado, N. Manjon, M. T. Cuevas, M. M. Thomson, L. Medrano, and R. Najera.** 2001. Detection and quantification of multiple drug resistance mutations in HIV-1 reverse transcriptase by an oligonucleotide ligation assay. J. Hum. Virol. **4:**238–248.
51. **Wainberg, M. A.** 2003. HIV resistance to nevirapine and other non-nucleoside reverse transcriptase inhibitors. J. Acquir. Immune Defic. Syndr. **34**(Suppl. 1)**:**S2–S7.
52. **Wallis, C. L., I. Mahomed, L. Morris, T. Chidarikire, G. Stevens, N. Rekhviashvili, and W. Stevens.** 2005. Evaluation of an oligonucleotide ligation assay for detection of mutations in HIV-1 subtype C individuals who have high level resistance to nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors. J. Virol. Methods **125:**99–109.

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