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# Optimization of the Oligonucleotide Ligation Assay, a Rapid and Inexpensive Test for Detection of HIV-1 Drug Resistance Mutations, for Non-North American Variants

**Ingrid A. Beck, MS**[*,†], **Claudia Crowell, MD**[*], **Robin Kittoe, BS**[*], **Helba Bredell, MS**[‡], **Molefe Machaba, Hons**[‡], **Carolyn Willamson, PhD**[‡], **Wouter Janssens, PhD**[§], **Sabelle Jallow, PhD**[§], **Guido van der Groen, PhD**[§], **Yiming Shao, MD, PhD**[||], **Mini Jacob, MD**[¶], **N. M. Samuel, MD**[¶], **Ivette Lorenzana de Rivera, MS**[#], **Nicole Ngo-Giang-Huong, PhD**[**], **Sharon Cassol, PhD**[††], **George Alemnji, PhD**[‡‡], and **Lisa M. Frenkel, MD**[*,†]

[*]*Divisions of Infectious Diseases and Virology, University of Washington, Seattle, WA* [†]*Seattle Children's Hospital Research Institute, Seattle, WA* [‡]*Department of Clinical Laboratory Sciences, University of Cape Town, Cape Town, South Africa* [§]*Institute of Tropical Medicine, Antwerpen, Belgium* [||]*National Center for AIDS/STD Control and Prevention, Beijing, China* [¶]*Department of Experimental Medicine, Tamil Nadu Dr. MGR Medical University, Chennai, India* [#]*Department of Microbiology, Universidad Nacional Autonoma, Tegucigalpa, Honduras* [**]*IRD URI 174/Program for HIV Prevention and Treatment, Chiang-Mai, Thailand* [††]*MRC Unit for Inflammation and Immunity, Department of Immunology, University of Pretoria, Pretoria, South Africa* [‡‡]*Faculty of Medicine and Biomedical Sciences, University of Yaounde, Yaounde, Cameroon*

## Abstract

**Objective—**We evaluated the feasibility of the oligonucleotide ligation assay (OLA), a specific, sensitive, and economical ligase-based point mutation assay designed to detect HIV-1 drug–resistance mutations at 12 codons of HIV-1 subtype B *pol*, for potential use in resource-poor settings.

**Methods—**Specimens from HIV-1–infected individuals collected by 7 international laboratories, including subtypes A, B, C, D, F, G, J, and recombinants AE and AG, were tested by the OLA developed for HIV-1 subtype B. Common polymorphisms that interfered with reactivity of the OLA were identified and modified probes designed and evaluated.

**Results—**92.5% (2410) of 2604 codons in specimens from 217 individuals were successfully genotyped by the subtype B OLA. A high rate (range 8.3%–31.2%) of indeterminate results (negative OLA reaction for both mutant and wild type) was observed for 5 codons. Modified probes at reverse transcriptase codons 151 and 184 and protease codon 90 increased the rate of valid OLA to 96.1%.

**Conclusions—**The OLA designed for HIV-1 subtype B genotyped most *pol* codons in non-B subtypes from Asia and Africa but was improved by addition of several modified probes. International laboratories experienced in molecular techniques were able to perform the OLA.

### Keywords

HIV drug resistance; HIV-1 non-B subtypes; resistance testing; oligonucleotide ligation assay; minor genotypes; point mutation assay; dried blood spots

Correspondence to: Lisa M. Frenkel, MD, 1900 Ninth Avenue, Seattle, WA 98101 (e-mail: lfrenkel@u.washington.edu).

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## INTRODUCTION

Antiretroviral therapy has been instrumental in improving the health of HIV-1–infected individuals and in the reduction of mother-to-child HIV-1 transmission. Drug-resistant viruses have been transmitted and selected during antiretroviral therapy.[1] Resistant variants have compromised the immunological benefits of antiretroviral therapy and interventions designed to reduce mother-to-child HIV-1 transmission.[1] Thus, monitoring drug resistance in the community and within the individual is recommended to optimize antiretroviral treatment.[1]

Consensus sequencing and consensus phenotype are the most commonly used assays for detecting drug-resistantHIV-1.[1] These tests are costly, making them impractical for large-scale studies and inaccessible to most resource-poor communities. We have developed a sensitive, specific, and inexpensive ligase-based point mutation assay, the oligonucleotide ligation assay (OLA), that detects mutations in HIV-1 *pol* conferring resistance to protease (PR) and reverse transcriptase (RT) inhibitors.[2–4] This high-throughput system uses differentially modified oligonucleotides specific for wild-type or mutant sequences and a type-common oligonucleotide. When hybridized to the DNA template [HIV-1 polymerase chain reaction (PCR) amplified from infected individuals' specimens], these oligonucleotides are covalently joined by a thermostable DNA ligase, allowing sensitive detection of both genotypes in a single well of a microtiter plate.[5] The current version of the OLA detects 15 mutations in 12 codons of HIV-1 subtype B *pol* associated with resistance to most currently FDA-approved antiretroviral drugs. The OLA primers and probes are available as a kit from the NIH AIDS Reference and Reagent Program (http://www.aidsreagent.org) for no-cost distribution to researchers. Because this test is rapid, inexpensive, and simple to perform and interpret, it is suitable for use in resource-limited settings. However, limited evaluation of the assay has been performed on HIV-1 variants collected outside North America, including non-B subtypes.

The specificity of the OLA for mutant versus wild-type virus is conferred by the ligation reaction and specifically by the requirement for the 2 bases flanking each side of the oligonucleotide junction be complementary to the target DNA for ligation.[6] Performing the OLA at relatively low temperatures minimizes the effects of polymorphisms within the target sequence, which are common in HIV-1 *pol*,[7,8] while maintaining the specificity at the site of interest. The OLA fails if the target has genetic polymorphisms within 2 bases of the ligation site or multiple polymorphisms within the region complementary to one of the OLA probes.[2,3] Because genetic variation could limit the use of the OLA, especially on HIV-1 non-B subtypes, one aim of this study was to evaluate the HIV-1 subtype B OLA probes on non-North American HIV-1 variants, including non-B subtypes, and modify the probes as needed to enable detection of the targeted mutations. A secondary aim was a preliminary assessment of implementing the OLA kits (available from the National Institutes of Health) in others' laboratories, especially in Africa and Asia.

## MATERIALS AND METHODS

### Subject's Specimens

Blood specimens were obtained from HIV-1–infected individuals living in Belgium (n = 44), Cameroon (n = 5), China (n = 30), Honduras (n = 39), India (n = 45), South Africa (n = 43), and Thailand (n = 20), after approval by each regional committee monitoring research in humans. Specimens consisted of whole blood collected as dried blood spots (DBS) on FTA (Whatman, Florham Park, NJ) (n = 84, Honduras and India) or 903 specimen collection (Whatman) filter paper (n = 15, Cameroon and Thailand), or cDNA derived from the plasma of clinical specimens after RNA extraction and reverse transcription (n = 127, Belgium, China, South Africa, and Thailand). The specimens from Belgium, China, and South Africa were tested by OLA in regional laboratories. PCR-amplified nucleic acids with invalid or

indeterminate OLA results in the regional laboratories were sent to the Seattle Laboratory for repeat testing. Blood specimens collected as DBS in Cameroon, Honduras, India, and Thailand were sent to Seattle and assayed only in Seattle.

### Processing of DBS Samples

DBS samples were collected, transported, and stored at room temperature. Before PCR amplification, 3-mm punches taken from whole blood specimens collected on FTA paper (Whatman) were washed following the manufacturer's instructions.[9] Specimens collected on 903 paper were extracted using chelex-100 (Bio-Rad, Hercules, CA). Briefly, a circle containing approximately 50 μL of whole blood was excised and washed twice with a solution of 0.1% Triton for 15 minutes. Then, 250 μL of 10% chelex-100 was added, incubated for 2 hours at 56°C, and then boiled for 15 minutes. The extracted DNA in the supernatant was used directly for PCR.

### Polymerase Chain Reaction

Both DNA bound to the FTA paper (1, 3-mm punch) and DNA extracted from the S&S 903 paper (10 μL) were amplified by nested PCR using first round primers PRA (CCTAGGAAAAAGGGCTGTTGGAAATGTGG) or IBF1 (AAATGATGACAGCATGTCAGGGAGT) and IBR1 (AACTTCTGTATATCATTGACAGTCCA), and second round primers PRB (ACTGAGAGACAGGCTAATTTTTTAGGGA) and IBR2 (CAAAGGAATGGAGGTTCTTTCTGATG). These primers were designed using sequences in the Los Alamos HIV-1 Sequence Database (Los Alamos National Laboratory, http://www.hiv.lanl.gov/content/hiv-db/PRIMALIGN/PRIME.html) to amplify a wide range of HIV-1 subtypes. Each round of PCR contained 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmol of each forward and reverse primer, and 2.5 U of *Taq* DNA polymerase. Cycling conditions consisted of an initial denaturation step of 94°C for 5 minutes, followed by 35 cycles of 94°C for 15 seconds, 55°C for 20 seconds, and 72°C for 2 minutes in first round or 1 minute in second round, and a final extension step of 72°C for 7 minutes. Plasma HIV-1 RNA was extracted using the QIAmp viral RNA kit (Qiagen, Valencia, CA), reverse transcribed with Expand Reverse Transcriptase (Roche, Brussels, Belgium) or ThermoScript RT-PCR System (Invitrogen Corp, San Diego, CA), and amplified with the same primers and conditions described above. The product amplified, a 1168-bp DNA fragment extending from HIV-1 *gag* to codon 228 of the RT gene, was visualized in a 1% agarose gel after electrophoresis and ethidium bromide staining. Only specimens yielding amplicons with a visible band of appropriate size were submitted to the OLA.

### Oligonucleotides for Ligation Detection

Ligation oligonucleotides specific for wild-type or mutant sequences[2–4] were modified at the 5′ end by addition of digoxigenin or fluorescein, respectively. Joining oligonucleotides complementary to common sequences adjacent to both wild-type and mutant codons were biotinylated at the 3′ end and phosphorylated at the 5′ end. Modified, high performance liquid chromatography-purified oligonucleotides were obtained from MWG-Biotech Inc (High Point, NC).

### Oligonucleotide Ligation Assay

The procedure and reaction conditions have been described[2,3] and a detailed protocol is found at http://depts.-washington.edu/idimmweb/faculty/frenkel/OLAmanual1305april04.pdf (OLA Manual, Version 1.4, July 2007). All specimens were analyzed using the reagents available in the subtype B OLA kits prepared and distributed by the NIH AIDS Reagents and Reference Program that evaluate mutations at codons 30, 50, 82, 84, 88, and 90 of HIV-1 PR

and codons 65, 103, 151, 181, 184, and 215 of HIV-1 RT. Samples with indeterminate results, defined as not reacting with either the wild-type or mutant OLA reagents, were retested with newly designed OLA probes only in the Seattle Laboratory. The HIV-1 mutant and wild-type controls for each codon (described previously[3]) included in each assay plate were used to determine whether the plate results were valid. All subjects' samples and controls were analyzed in duplicate. Optical densities at 490 nm (mutant genotype) and 450 nm (wild-type genotype) were measured in a Spectramax 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

### Sequencing

Residual PCR primers and dNTP were removed from the amplified DNA products by treatment with shrimp alkaline phosphatase and exonuclease I (PCR Product Pre-Sequencing Kit; Amersham Laboratories, Arlington Heights, IL). Cleaned PCR products were then directly sequenced using forward primers PRB and RT4[2] and reverse primers PR2[3] and IBR2, with fluorescence-labeled dideoxy chain terminators (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Foster City, CA) and a Prism 3730XL Genetic Analyzer (Applied Biosystems). Sequences encoding HIV-1 PR and RT were assembled and edited using Sequencher 4.5 (Gene Codes Corp, Ann Arbor, MI).

### HIV-1 Subtype Determination

The HIV-1 subtype of the different specimens was determinted by analysis of the specimens' sequences submitted to the Stanford University HIV-1 Database (http://hivdb.stanford.edu), Env and Gag heteroduplex mobility assay[10,11] or by epidemiologic prevalence.

## RESULTS

Blood specimens from 217 HIV-1–infected individuals residing in Belgium (n = 44), Cameroon (n = 5), China (n = 30), Honduras (n = 36), India (n = 39), South Africa (n = 43), or Thailand (n = 20) were evaluated for 15 drug-resistance mutations in 12 codons of HIV-1 *pol* by the OLA. The specimens included HIV-1 subtypes A, B, C, D, F, G, J, and recombinants AE and AG (Table 1). The mutations assayed were the nucleotides that most frequently encode D30N, I50V, V82A/S/T, I84V, N88D, and L90M in HIV-1 PR and K65R, K103N, Q151M, Y181C, M184V, and T215Y/F in HIV-1 RT.

Specimens collected in Cameroon, Honduras, India, and Thailand were assayed in the Seattle Laboratory whereas specimens collected in Belgium, China, and South Africa were assayed locally in their regional laboratories. The OLA data generated in the international laboratories were reviewed and interpreted in Seattle. The OLA was valid for 77.3% of 1069 codons analyzed regionally. In all, 243 codons had invalid OLA results due to technical factors (controls suboptimal on the plate or replicates with discrepant results). PCR amplicon from the latter and from codons with indeterminate genotypes (negative OLA reaction for both mutant and wild type) was sent to Seattle for repeat testing. All valid OLA results obtained in Seattle and international laboratories were combined in the final analysis.

### Performance of the PCR Primers

Of 127 plasma specimens collected in Belgium, China, South Africa, and Thailand, 100% amplified with the primer sets PRA/IBR1 and PRB/IBR2 (n = 64/64) or IBF1/IBR1 and PRB/IBR2 (n = 63/63). However, only 77 (77.8%) of 99 specimens collected as DBS in Honduras, India, Cameroon, and Thailand amplified when initially tested with primers PRA/IBR1 and PRB/IBR2. Nineteen of those that failed amplification were tested again with the first round forward primer PRA replaced by IBF1, which amplified 13, suggesting that the primer IBF1 performed better on non-B specimens. The 9 DBS specimens that failed amplification were

not evaluated for PCR inhibitors, degradation of the nucleic acid, or the HIV-1 DNA content. Because a PCR amplicon is required to perform the OLA, these specimens were not tested by OLA and not included in the analysis of the OLA.

### Performance of the Ligation Oligonucleotides

A total of 2604 codons were genotyped by the subtype B OLA reagents of which 92.5% (2410) were successfully evaluated (Table 1). The remaining 194 codons had indeterminate results, defined as a negative OLA reaction for both mutant and wild type resulting from failure of the probes to either hybridize or ligate. High rates of indeterminate OLA were observed at RT codons 184 (31.2%) and 151 (8.3%) and PR codons 88 (13.8%), 90 (10.1%), and 82 (8.3%). The indeterminate results at codons 184, 88, and 90 were primarily in subtype C viruses whereas those testing indeterminate at codons 151 and 82 were distributed among multiple subtypes. Lower rates of indeterminate results were observed in RT codons 65 (5.1%), 181 (5.1%), and 103 (3.7%). And the fewest indeterminate results were at PR codons 30 (0.9%), 50 (0%), and 84 (1.4%) and RT codon 215 (1.4%). Genetic polymorphisms were the likely cause of assay failure; therefore, specimens with indeterminate OLA results were sequenced, and when common polymorphisms were identified, new probes were designed and tested.

### Optimization of the Ligation Oligonucleotides for Non-B HIV-1 Subtypes

A review of HIV-1 *pol* sequences from a panel of non-B HIV-1 clones,[12] from subjects with non-B subtypes residing in the Seattle area (n = 17, data not shown) and from the Los Alamos HIV-1 Sequence Database, allowed us to identify single-base polymorphisms of moderate prevalence within 2 bases of the ligation site of the oligonucleotide probes. Probes complementary to polymorphisms in RT codons 151, 181, and 184 (Table 2) were synthesized and evaluated in specimens that tested indeterminate with the subtype B probes. The modification of the probes for M184V at codon 183 (TAC → TAT) decreased the rate of indeterminate results from 31.2% to 2.7% across subtypes and from 65.9% (60/91) to 3.3% (3/91) for subtype C (Table 1). The modified oligonucleotide probe for codon 151, complementary to a polymorphism CAG to CAA at this codon, reduced the rate of indeterminate tests from 8.3% to 0.9%. A third probe with the first nucleotide of the common probe for RT codon 181 modified from T to C only modestly reduced the rate of indeterminate tests from 5.1% to 4.1%.

Specimens that had indeterminate test results with the subtype B probes, or remained indeterminate after evaluation with the modified OLA probes, were subsequently sequenced to identify additional interfering polymorphisms. A polymorphism 4 bases from the ligation site on the genotype-specific probe and several others in the region of the common probe were frequently detected in sequences of the specimens with indeterminate results at codon 90 of PR, especially those with HIV-1 subtype C. Modified probes for L90M that included these base changes (Table 2) were evaluated and decreased the indeterminate rate from 10.1% to 4.1% (Table 1).

The specimens that tested indeterminate with the subtype B and the modified oligonucleotides for codons 151, 181, 184, and 90 had sequences that were not complementary to the probes (Table 3). Of note, the polymorphism incorporated into the modified 181 common probe was not prevalent among the specimens we tested, and when observed, this mutation occurred with several additional polymorphisms in the region of both the genotype-specific and the common probes that precluded their ligation. Also of interest, a polymorphism 2 bases from the ligation site of RT codon 103 (AAA → AGA) encoded a K103R mutation that has not been associated with resistance[13] (Table 3) and was observed only in subtype B specimens from Honduras. Indeterminate reactions at PR codons 82 and 88 included polymorphisms within 2 nucleotides of the ligation site in a subset of specimens and multiple other polymorphisms in the remaining

samples (Table 3). Modified oligonucleotide probes were not tested for RT codon 103 or PR codons 82 and 88 due to the relatively low prevalence of particular interfering polymorphisms and heterogeneity in the sequences in regions corresponding to the OLA probes.

### Comparison of Genotypes Detected by OLA and Consensus Sequencing

Consensus sequences generated in the Seattle Laboratory for specimens with indeterminate OLA results (n = 48 specimens for PR, n = 37 specimens for RT) were compared with the genotypes obtained by OLA (excluding the one codon per subject that tested indeterminate). Genotypes were concordant for 408 (96%) of the 425 codons analyzed. The discordant genotypes (4%) corresponded to mixtures of wild-type and mutant virus detected by the OLA when consensus sequencing detected only wild-type variants. No drug-resistant genotypes detected by sequencing were missed by the OLA.

## DISCUSSION

The utility of the OLA, a sensitive, simple, and economical assay, designed for the detection of HIV-1 drug–resistance mutations in subtype B US variants,[2,3,14] was demonstrated for a diverse array of HIV-1 subtypes collected outside North America. In addition, 3 international laboratories experienced with molecular techniques were able to implement the OLA following the kit instructions.

In this study, we demonstrated that reagents developed to detect primary drug-resistance mutations in HIV-1 subtype B prevalent in the United States can effectively assay most (89%–93%) viruses of non-B subtypes and nearly all (97%) B subtypes from Central America, Asia, and Europe. Adding several modified reagents increased the reactivity of the OLA to 97% of RT and 95.2% of PR codons in specimens contributed by 7 sites in Africa, Asia, Central America, and Europe. Although these assay performance rates may not be representative of an extensive sampling of global HIV-1 variants and subtypes, our study demonstrates several attributes of the OLA: First, the available reagents genotype the majority of codons associated with high-level HIV-1 resistance to most nucleoside reverse transcriptase inhibitors, nonnucleoside reverse transcriptase inhibitors, and protease inhibitors currently in use.[1] Second, the subtype B reagents have a high rate of reactivity across most non-B HIV-1 subtypes. Third, publicly available databases and consensus sequences of regional specimens can be used to modify the reagents to increase the OLA reactivity for most codons. Similarly, sequence data can be used to develop reagents for prevalent regional genotypes, such as K103R in RT that was prevalent among Honduran specimens, or additional codons, as we have done for RT codons 106 and 190 (Beck IA, April 2007 and June 2004). Fourth, the OLA detects minority mutant genotypes that are not apparent when the same PCR amplicon is evaluated by consensus sequencing.[14]

Among the specimens tested, the subtype B OLA failed most frequently at PR codons 82, 88, and 90 and at RT codons 151 and 184, with indeterminate rates ranging from 8.3% to 31.2%. Other codons had indeterminate rates of 5% or less, similar to or only slightly higher than the 2%–3.5% observed in specimens from ARV-naive (Ellis GM, June 2007) and highly treated North Americans infected with HIV-1 subtype B.[2,3] Interfering genetic polymorphisms, especially those located within 2 bases of the ligation site, are the most common cause of assay failure.[2,3] Single-nucleotide modifications within 2 bases of the ligation site of the probes for RT codons 151 and 184 and changing several nucleotides in the probes for PR codon 90 restored the reactivity of the OLA probes for most specimens we evaluated with initial indeterminate results. However, heterogeneity in the target sequences at other codons precluded simple modifications to improve the performance of several probes. It is possible that the use of mixed bases or promiscuous nonstandard nucleotides, such as deoxyuridil or deoxyinosine, may improve the OLA performance at the highly polymorphic regions probed for PR codons 82

and 88 and RT codons 65 and 181. This approach could be explored for the polymorphisms described in this study and sequence databases to create ‘‘universal probes’’ for specimens regardless of HIV-1 subtype. We plan on exploring these modifications and to include the optimized probes to the next version of the OLA kits available from the NIH AIDS Reagent and Reference Program.

Other groups have used the OLA for drug-resistance genotyping. Rates of indeterminate results were similar to our present study when testing subtype B and non-B viruses from Spain.[15] Testing of RT codons 65, 103, 151, 181, 184, and 215 in HIV-1 subtype C South African specimens[16] had an overall low reactivity rate due to high genetic variation in the region of the OLA probes. In our cohort, the highest rates of indeterminate OLA results were observed among subtype C specimens at RT codon 184 (66%) and PR codons 88 (19.8%) and 90 (17.6%). The modified probes for codons 184 and 90 reduced the indeterminate results for subtype C specimens to 3.3% at both codons, demonstrating that optimization of OLA probes can enable genotyping of specific resistance mutations in subtype C viruses. In addition, a recent report on the development of an OLA for detection of Q151M and M184V in HIV-2 infections[17] shows that virus-specific primers and OLA probes will be needed to evaluate certain drug-resistance mutations.

Multiple assays designed to detect point mutations in HIV-1 have been developed for the evaluation of minor populations of drug-resistant HIV-1.[18–23] These assays vary in the sensitivity and specificity of detecting mutations and in the required laboratory equipment and technical expertise. Few have reported performance across HIV-1 subtypes, although modifications in each assay’s reagents should allow the detection of targeted mutations. The line probe assay[18] and mutagenically separated PCR assay[19] have reported variable reactivity for non-B specimens.[19,24] Both the LigAmp assays, which utilize DNA ligase for specificity, similar to the OLA, and real-time PCR[21] and a selective mutation-specific PCR real-time assay,[22] have been shown to be very sensitive for the evaluation of nevirapine-resistance mutations K103N and Y181C in women infected with HIV-1 subtypes A, C, and D.[22,25,26] These real-time PCR-based assays seem to require specific primers/probes for each subtype, in addition to more expensive equipment and reagents compared with the OLA, that might limit use for the screening of multiple mutations in resource-limited communities. An allele-specific real-time PCR developed for multiple drug-resistance codons[23] has not yet been modified for non-B subtypes. Parallel allele-specific sequencing[27] is a new primer extension modality that detects minor genotypes, including linked mutations, that likewise, has not been modified for non-B subtypes.

Screening for all resistance-associated mutations by a point mutation assay such as the OLA is impractical. Therefore, the OLA kits were designed to evaluate only mutations associated with high-level resistance to FDA-approved antiretrovirals. As the guidelines for surveillance of drug-associated resistance mutations in HIV-1–infected individuals have been modified since the development of the subtype B OLA,[28–30] we have developed probes for detection of the G190A mutation in RT associated with resistance to nonnucleoside reverse transcriptase inhibitor and V106M (OLA probes for Drug Resistance Mutations in HIV-1 *pol*; http://depts.washington.edu/idimmweb/facultyMember.php?sort=Frenkel), a mutation frequently detected in nonnucleoside reverse transcriptase inhibitor–treated individuals infected with subtype C.[31,32] V82I, a PR mutation not currently detected by the OLA assay, has not been associated with drug resistance; however, it has been described as a frequent mutation in subtype G viruses of treatment-naive persons.[33] As only one nucleotide change is required to mutate to 82T or 82M, it may be useful to design probes for the detection of V82I when analyzing specimens of subtype G variants.

Similar to previous comparisons, OLA and consensus sequence genotypes derived from the same amplicon demonstrated concordance. Except consistent with past evaluations,[2,3,14] the OLA seemed more sensitive compared with consensus sequencing for the detection of minor mutant variants in mixed viral populations.

In addition to performing with high sensitivity and specificity, the OLA is simple and has a high throughput and a low cost. Utilizing only a thermocycler and a spectrophotometer, one codon can be genotyped in 43 specimens on a single 96-well plate in less than 5 hours, with OLA reagents costing less than US $1.50/codon/specimen. As part of this project, several international laboratories that had not previously used the OLA tested their specimens. The majority of OLA plates performed well. However, occasionally the controls were inadequate (low optical density on the positive controls or high background in the negative controls), possibly due to degradation of oligonucleotides or other problems with reagents. OLA results in these plates were considered invalid and were repeated in Seattle. Although not evaluated as part of this project, our collaborative work has shown that experienced international laboratories can perform the OLA, but as with other molecular techniques, the successful implementation of the assay requires validation of reagents, procedures, and equipment to optimize the assay in each laboratory.

Nucleic acids amplified from plasma HIV-1 RNA and proviral DNA, including specimens collected on filter paper, were genotyped by OLA. Of note, the sensitivity of the OLA, and especially for detection of minority mutant genotypes, may be diminished when evaluating specimens collected on filter paper, as suggested by a 9.1% (9/99) failure of PCR to amplify virus from DBS collected for this study. The decreased sensitivity is likely due to the small volume of blood collected and consequently low HIV-1 DNA copy number,[34] the presence of PCR inhibitors, or DNA degradation.

Other important limitations of the OLA are that novel mutations are not identified and only a selected number of resistance mutations are evaluated. Sequencing, on the other hand, gives comprehensive information on all the possible mutations within the region probed. Sequencing may be preferred for genotyping of specimens from highly drug-experienced individuals, with the caveat that mutations at low levels may not be detected. In contrast, the OLA, with a higher sensitivity for detection of low-level mutations, high throughput, low cost, and simplicity, may be of great utility for the surveillance of drug resistance in settings where the antiretrovirals mutational pattern is predictable, such as antiretroviral roll-out programs adopted by developing countries recently offering antiretroviral treatment[35] and the various programs implemented for the prevention of mother-to-child transmission of HIV-1.[36]

In summary, the OLA is a sensitive, rapid, economical, and simple test for the detection of primary drug-resistance mutations in HIV-1 subtype B and non-B subtypes prevalent in Asia and Africa. The OLA is easily implemented in laboratories experienced in molecular techniques and can be adapted to evaluate resistance mutations in HIV-1 variants circulating in a particular geographical region. Therefore, as more antiretroviral agents become available in resource-limited areas, the OLA could be a valuable tool to monitor HIV-1 drug resistance.

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**TABLE 1**

Evaluation of Drug-Resistance Mutations in 217 Blood Specimens With Diverse HIV-1 Subtypes Using OLA Reagents Designed for Subtype B and Repeated Using Reagents Modified to Improve Reactivity of the Assay Across Subtypes

| HIV-1 Subtype | No. Specimens Evaluated | No. (*) Specimens Indeterminate by OLA at Codons: RT 65 | RT 103 | RT 151 | RT 181 | RT 184 | PR 215 | PR 30 | PR 50 | PR 82 | PR 84 | PR 88 | PR 90 | Total % (^) of Indeterminate Codons |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 6 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 3 | 1 | 9.7 |
| B | 70 | 3 | 5 | 7 (0) | 2 (1) | 1 (0) | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 2.5 (1.4) |
| C | 91 | 5 | 1 | 7 (1) | 2 | 60 (3) | 0 | 0 | 0 | 7 | 1 | 18 | 16 (3) | 10.7 (3.8) |
| D | 5 | 1 | 0 | 1 (0) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 6.7 (5.0) |
| F | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 8.3 |
| G | 8 | 0 | 0 | 2 (0) | 1 | 2 | 0 | 2 | 0 | 3 | 0 | 1 | 0 | 11.4 (9.3) |
| J | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 8.3 |
| AE | 25 | 1 | 1 | 1 | 3 (2) | 1 (0) | 0 | 0 | 0 | 6 | 1 | 4 | 2 | 6.7 (6.0) |
| AG | 8 | 0 | 0 | 0 | 3 | 4 (1) | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 10.4 (7.3) |
| Total number for all subtypes | 217 | 11 | 8 | 18 (2) | 11 (9) | 68 (6) | 3 | 2 | 0 | 18 | 3 | 30 | 22 (9) | |
| % indeterminate with subtype B probes | | 5.1 | 3.7 | 8.3 | 5.1 | 31.2 | 1.4 | 0.9 | 0 | 8.3 | 1.4 | 13.8 | 10.1 | 7.5 |
| % indeterminate with modified probes** | | — | — | 0.9 | 4.1 | 2.7 | — | — | — | — | — | — | 4.1 | 3.9 |

* Number of indeterminate samples remaining after testing with modified oligonucleotides.

^ Percent of indeterminate samples remaining after testing with modified oligonucleotides.

** Modified oligonucleotide probes were synthesized and tested only for RT codons 151, 181, and 184 and PR codon 90.

**TABLE 2**

Sequence of Oligonucleotide Probes for HIV-1 Subtype B OLA and Modifications Made for Detection of Drug-resistance Mutations in Non-B Subtypes

| HIV-1 pol region | Mutation Detected | Oligonucleotide Probes | Sequence: Genotype-Specific* Common† |
|---|---|---|---|
| PR | L90M | WT‡ | CAACATAATTGGAAGAAATCTGT* TGACTCAGATTGGTTGCACTTT |
| | | Mutant | ----------A* ---------- |
| | | Modified§ | ----------**A**---* ----------**C**----**A**------**A**- |
| RT | Q151M | WT | CAGTACAATGTGCTTCCACA* GGGATGGAAAGGATCAC |
| | | Mutant | ----------AT* ---------- |
| | | Modified | ----------* **A**---------- |
| | Y181C | WT | ACAAAATCCAGACATAGTTATCTA* TCAATACATGGATGATTTGTATGTA |
| | | Mutant | ----------G* ---------- |
| | | Modified | ----------* **C**---------- |
| | M184V | WT | AGACATAGTTATCTATCAATACA* TGGATGATTTGTATGTAGGATC |
| | | Mutant | ----------G* ---------- |
| | | Modified | ----------**T-*** ---------- |

*Indicates the ligation site

†Bases comprising the codons of interest are shaded

‡WT, wild-type

§Nucleotide modifications are in boldface type

## TABLE 3

Nucleotide Sequences of OLA Probes and the Specimens With Indeterminate Genotype by OLA, Showing the Region Complementary to the Probes on Either Sides of the Ligation Site

| HIV-1Subtype | RT Codon/Specimen | Results of Direct Sequencing (5′ → 3′) |
|---|---|---|
| Con B | Codon 65 wt | CTCCAGTATTTGCCATAAAGAA* RAAAGACRGTACTAAATGGAGAA |
| | Codon 65 mut | ----------G* ---------- |
| B | Dzy8 | ----------G----* A--GA--A------G--------- |
| B | Dzy17 | -----A----------C---* A--GA-TA------G--------- |
| B | Dzy19 | -----A----------* G--GA--A--GA--G--------- |
| C | Efc32 | -----A----------T----A---* G--G---A-------G-------- |
| C | Efc38 | -----A----------A---* G--GA--A------GG-------- |
| C | Efc50 | -----A----------A----A---* G--G---A-------G-------- |
| C | Ena9 | -----A----------GA---* G--G---A-------G-------- |
| D | Dzy1 | -----A----------* A--GA--A------GG-------- |
| F | Dzy7 | ----------T----------* A-----AA--T-C-GT-CT--AGT |
| AE | Dxy39 | -----A------T-----G-----* A--G---A-C--C---------G- |
| Con B | Codon 103 wt | ACATCCCGCAGGGTTAAAAAAGAAA* AAATCAGTAACAGTACTGGATGTGGGT |
| | Codon 103 mut | ----------C* ---------- |
| A | Dzy30 | G-----A--G—T----------G* ----------A---------G |
| B | Dub35 | ---------G---C--C------G-* ---------- |
| B | Dtr80 | ------T----------G* ---------- |
| B | Dtx67 | ----------G-* ----------R--- |
| B | Duf36 | ----------G--G-* ----------A---------- |
| B | Duf37 | ----------G-* ----------A |
| AE | Dzy31 | G-----A--G—T---G--C-----* ----------A---------G |
| Con B | Codon 151 wt | CAGTACAATGTGCTTCCACA* GGGATGGAAAGGATCAC |
| | Codon 151 mut | ----------AT* ---------- |
| | Codon 151 mod | ----------* A---------- |
| AE | Dxy35 | ----------G--G--* ---------- |
| C | Ena31 | --A—T--------G-----* ---G---------- |
| Con B | Codon 181 wt | ACAAAATCCAGACATAGTTATCTA* TCAATACATGGATGATTTGTATGTA |
| | Codon 181 wt | ----------G* ---------- |
| | Codon 181 mod | ----------* C---------- |
| B | Dtx74 | ----------A----G-AW--T--* ---------- |
| C | Ena28 | -AG---------AT----C--T--* ------T--------RC-------- |
| C | Ena45 | ----------A-----C—T-G* ------TG--------C-------- |
| G | Dzy40 | -A----------A--G--G-----* C--G---G----------A------ |
| AE | Esp1 | -A----------A--G-----T--* ----------C-A------ |
| AE | Esp8 | -A----------A--G-----T--* ----------C-------- |
| AG | Dzy3 | -A----C-----G--G—G-----* C-----TG----------A------ |
| AG | Dzy45 | -A----------AC-G—G-----* -------G----------A------ |
| AG | Eib35 | -A-T--------G-----G--T--* C-----T----------A------ |
| Con B | Codon 184 wt | AGACATAGTTATCTATCAATACA* TGGATGATTTGTATGTAGGATC |
| | Codon 184 mut | ----------G* ---------- |
| | Codon 184 mod | ----------T-* ---------- |
| C | Ena45 | ---A-----C--T-G------TG* -------C----------C--- |
| C | Efc38 | ---A-----C----------TG* -A-----C----------C--- |
| C | Efc54 | ---A-----C--------G—T-* -A-----C----------C-- |
| G | Dzy14 | ---AG-------T-G--------* ----------A------GC--- |
| G | Dzy40 | ---A--G--G-----C--G---G* ----------A---------- |
| AG | Eib35 | ---G-----G--T--C------T* ----------A---------- |
| Con B | Codon 215 wt | CAACATCTGTTGAGGTGGGGATTTAC* CACACCAGACAAAAAACATCAGAA |
| | Codon 215 mut F | ----------TT* ---------- |
| | Codon 215 mut Y | ----------TA* ---------- |
| A | Dzy30 | GCT-----A--A--A-----GC----* T----------G-------- |
| B | Dzy17 | --G-------G--A------G--CTT* ---------AC----G |
| B | Epw1 | ----------G-------G-----* ---------- |

| HIV-1 Subtype | PR Codon/Specimen | Results of Direct Sequencing (5′ → 3′) |
|---|---|---|
| Con B | Codon 30 wt | TATTAGATACAGGAGCAGATG* ATACAGTATTAGAAGAAATGAAT |
| | Codon 30 mut | ----------A* ---------- |
| G | Dzy14 | ----------* --------C--AC---C--AG-- |
| G | Dzy15 | ----------* --------C--AC---C--AG-- |
| Con B | Codon 82 wt | TATTAGTAGGACCTACACCTGT* CAACATAATTGGAAGAAATCTGT |

| HIV-1Subtype | RT Codon/Specimen | Results of Direct Sequencing (5′ → 3′) |
|---|---|---|
| | Codon 82 mutA | ----------C* ---------- |
| | Codon 82 mutS | ----------AG* ---------- |
| | Codon 82 mutT | ----------AC* ---------- |
| A | Dzy2 | ----G----------TC* ----------G---A--- |
| B | Dtx34 | -----A----R--C------CA-* T---------- |
| C | Ena3 | -----A----G----------* ----------G---A--- |
| C | Ena26 | ----------G---------C--* ----------CA--- |
| C | Ena42 | -R---A----G---------C--* ----------G-A--- |
| C | Eek36 | ----------GAC--------* ----------G-A--- |
| C | Eew44 | -------G-----C--------* ----------C------A--- |
| C | Edw35 | -------RR----C--------* T----------G-A--- |
| C | Esp7 | -------G-----C--------* ----------A--- |
| G | Dzy23 | ----------A-* ----------G------A--- |
| G | Dzy44 | --C----------A-* ----------A--- |
| G | Dzy40 | --C----------A-* ----------G------A--- |
| AE | Dzy31 | ----G--------T-C---C--* ----------C-----CA--- |
| AE | Dyf31 | ----------A--* ----------C-----A--- |
| AE | Dye8 | -G----------A-* ----------C-----A--- |
| AE | Esp1 | ----------C--G------* ----------C-----A--- |
| AE | Esp8 | ----------C--G------* ----------C-----R--- |
| AE | Esp10 | ----------C--G------* ----------C-----A--- |
| Con B | Codon 84 wt | AGGACCTACACCTGTCAACA* TAATTGGAAGAAATCTGTTGACT |
| | Codon 84 mut | ----------G* ---------- |
| C | Ena43 | G--R--------A-----T-* ----------A-------- |
| AG | Dzy38 | ----------C-----* --------C-G--CG-RA----C |
| Con B | Codon 88 wt | CCTGTCAACATAATTGGAAGAA* ATCTGTTGACTCAGATTGGTTG |
| | Codon 88 mut | ----------G* ---------- |
| A | Dzy2 | ---TC----------G-* --A---------- |
| A | Dzy12 | ----------G-* --A---------- |
| A | Dzy30 | ---A----------* --G-------C-----G--A-- |
| C | Dzy24 | ----------* --A----------AC----A-- |
| C | Dzy25 | ----Y----R---------G-* ----------Y----A-- |
| C | Dzy29 | ----C-------G---------* --T--A--------T-A--A-- |
| C | Ena2 | ----------G-* --A----------C----A-- |
| C | Ena21 | ---------R-------G----* --A----------C----A-- |
| C | Ena26 | --C----------* -CA----------C----A-- |
| **HIV-1Subtype** | **RT Codon/Specimen** | **Results of Direct Sequencing (5′ → 3′)** |
| C | Ena28 | ----------G-* -CA----------C----A-- |
| C | Ena34 | ----------G-* --A----------C----A-- |
| C | Ena39 | ----------G----* --A----------AC----A-- |
| C | Ena42 | --C----------G-* --A----------C----A-- |
| C | Ena8 | -----Y----------* -CA----A------C----A-- |
| C | Ena29 | ------R----------* -CA----------C----G-- |
| C | Edw35 | -----T----------G-* --A----------C--R-A-- |
| C | Eek36 | ----------G-* --A----------C----G-- |
| C | Eew56 | ----------G-* --T----------C----A-- |
| C | Esp7 | ----------* --A----------C----A-- |
| D/F | Dzy11 | ----C----R----------* --T----A--C--A-----C-- |
| F | Dzy34 | ----C-------G---------* --T--A-------T--A-A-- |
| G | Dzy13 | ---------G-------G----* --A--A-------A-------- |
| J | Eig31 | -----T----------* -CA----A-----A-----A-- |
| AE | Dzy31 | --C----------C----* -CA----------C-- |
| AE | Esp1 | ----------C----* --A----------C-------- |
| AE | Esp8 | ----------C----* --R----------C-------- |
| AE | Esp10 | ----------C----* --A----------C-------- |
| AG | Dzy38 | ----C----------C-G--* -CG-RA----C---------- |
| Con B | Codon 90 wt | CAACATAATTGGAAGAAATCTGT* TGACTCAGATTGGTTGCACTTT |
| | Codon 90 mut | ----------A* ---------- |
| | Codon 90 mod | ----------A---* --------C----A-----A-- |
| A | Dzy27 | ---------W---------A-AC* ----------T----- |
| C | Edw35 | T----------G---A---* -A------C--R-A-----A-- |
| D/F | Dzy11 | ----R----------T---* -A--C--A-----C-------- |
| J | Eig31 | T----------CA---* -A-----A-----A--T----- |
| AE | Dye1 | ----------C-----A---* --R--M----------T----- |
| AE | Dxy34 | ----------C-----A--A* ----------T---- |

| HIV-1Subtype | RT Codon/Specimen | Results of Direct Sequencing (5′ → 3′) |
|---|---|---|
| AG | Dzy38 | ----------C-G--CG-RA* ----C----------T----- |

Con B, consensus B; wt, wild type; mut, mutant.

* Ligation site.