Performance Characteristics of the TRUGENE HIV-1 Genotyping Kit and the Opengene DNA-Sequencing System

Daniel R. Kuritzkes,¹ Robert M. Grant,² Paul Feorino,³ Marshal Griswold,⁴ Marie Hoover,⁵ Russell Young,¹ Stephen Day,³ Robert M. Lloyd, Jr.,³ Caroline Reid,⁶ Gillian F. Morgan,⁶ and Dean L. Winslow⁶

Division of Infectious Diseases, University of Colorado Health Sciences Center, Denver, Colorado;¹ Gladstone Institute of Virology and Immunology, San Francisco;² and Consolidated Laboratories, Van Nuys, California;³ Visible Genetics, Inc., Suwanee, Georgia;⁴ Advanced BioMedical Laboratories, Cinnaminson, New Jersey;⁵ and Visible Genetics, Inc., Toronto, Ontario, Canada⁶

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The routine use of genotypic resistance testing requires convenient assays with high reproducibility that can be performed by laboratories skilled in molecular diagnostic techniques. The TRUGENE HIV-1 Genotyping Kit and OpenGene DNA Sequencing System are designed to provide bidirectional sequencing of the protease (PR)- and reverse transcriptase (RT)-coding regions of human immunodeficiency virus type 1 (HIV-1) pol. Studies were undertaken to determine the accuracy of this assay system in detecting resistance-associated mutations and to determine the effects of RNA extraction methods, anticoagulants, specimen handling, and potentially interfering substances. Samples were plasma obtained from HIV-infected subjects or seronegative plasma to which viruses derived from wild-type and mutant infectious molecular clones (IMC) of HIV-1 were added. Extraction methods tested included standard and UltraSensitive AMPLICOR HIV-1 MONITOR, QIAGEN viral RNA extraction mini kit, and QIAGEN Ultra HIV extraction kit, and NASBA manual HIV-1 quantitative NucliSens. Sequence data from test sites were compared to a “gold standard” reference sequence to determine the percent agreement. Comparisons between test and reference sequences at the nucleotide level showed 97.5 to 100% agreement. Similar results were obtained regardless of extraction method, regardless of use of EDTA or acid citrate dextrose as anticoagulant, and despite the presence of triglycerides, bilirubin, hemoglobin, antiretroviral drugs, HIV-2, hepatitis C virus (HCV), HBV, cytomegalovirus, human T-cell leukemia virus type 1 (HTLV-1), or HTLV-2. Samples with HIV-1 RNA titers of ≥1,000 copies/ml gave consistent results. The TRUGENE HIV-1 Genotyping Kit and OpenGene DNA Sequencing System consistently generate highly accurate sequence data when tested with IMC-derived HIV and patient samples.

The clinical significance of drug resistance in human immunodeficiency virus type 1 (HIV-1) infection is well-established (3, 13, 14). Patients treated with drugs to which their HIV-1 isolates are predicted to be resistant on the basis of genotypic or phenotypic assays are significantly less likely to achieve a virological response (4, 17). Conversely, use of drugs to which the virus is predicted to be susceptible enhances the likelihood of reestablishing virological control in patients in whom prior treatment regimens have failed (5). Randomized clinical trials demonstrate the clinical utility of drug resistance testing as a guide to the selection of subsequent antiretroviral regimens for patients with virological failure of their current regimen (1, 2, 7, 16). Guidelines recommending drug resistance testing in managing failure of antiretroviral therapy have been proposed by several expert panels (8, 10; see also guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents [http://www.atis.org]).

The routine use of genotypic resistance testing requires convenient assays with high reproducibility that can be performed by laboratories skilled in molecular diagnostic techniques. The TRUGENE HIV-1 Genotyping Kit and OpenGene DNA Sequencing System are designed to provide bidirectional sequencing of the protease (PR)-coding region (codons 10 to 99) and codons 41 to 237 of the reverse transcriptase (RT)-coding region of HIV-1 pol. A detailed description of this system is described in the accompanying work (9). In order to determine the effects of specimen characteristics on the performance characteristics of the TRUGENE HIV-1 Genotyping Kit and the OpenGene DNA Sequencing System, a series of studies was undertaken to assess the effect of RNA extraction methods, anticoagulants, sample freezing and thawing, and the presence of potentially interfering substances. Ability to detect drug resistance-associated mutations was also evaluated using a panel of plasma samples spiked with HIV-1 derived from infectious molecular clones carrying a variety of resistance-associated mutations in PR and RT.

MATERIALS AND METHODS

Plasma samples. Plasma from HIV-1-infected subjects was collected by routine methods or by plasmapheresis. Specimen collection was conducted using protocols that complied with all relevant federal guidelines and that were approved by the appropriate institutional review boards. All subjects gave signed informed consent. HIV-1-negative human plasma was purchased from a commercial source (Boston Biomedica, Inc., Bridgewater, Mass.).

Viruses. Wild-type and mutant viruses were prepared from infectious molecular clones of HIV-1 or obtained from plasma samples collected from HIV-infected subjects by plasmapheresis as described elsewhere (9). Mutant variants of HIV-1 were prepared by site-directed mutagenesis of an infectious molecular clone of HIV-1LAI (pLAI2) (kindly provided by L. Montagnier through the NIH AIDS Research and Reference Reagent Program). Mutations were introduced into the PR- or RT-coding region of pol using the Altered Sites II in vitro mutagenesis system (Promega, Madison, Wis.). The resulting plasmids were linearized by restriction enzyme digestion and electroporated into CEM-SS cells.

¹ Corresponding author. Present address: Partners AIDS Research Center, Brigham and Women’s Hospital, 65 Landsdowne St., Rm. 449, Cambridge, MA 02139. Phone: (617) 768-8371. Fax: (617) 768-8378. E-mail: dkuritzkes@partners.org.
Table 1. Resistance-associated mutations in HIV-1 PR and RT present in test samples

<table>
<thead>
<tr>
<th>Isolate or patient</th>
<th>Source</th>
<th>PR</th>
<th>RT</th>
<th>Plasma HIV-1 RNA level (copies/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VA-MH-001</td>
<td>Plasma</td>
<td>63P</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>VA-MH-003</td>
<td>Plasma</td>
<td>None</td>
<td>184V</td>
<td>2,618</td>
</tr>
<tr>
<td>VA-MH-004</td>
<td>Plasma</td>
<td>None</td>
<td>67N, 7OR, 103N, 184V, 219Q</td>
<td>1,279</td>
</tr>
<tr>
<td>VA-MH-007</td>
<td>Plasma</td>
<td>36I</td>
<td>None</td>
<td>28,881</td>
</tr>
<tr>
<td>VA-MH-009</td>
<td>Plasma</td>
<td>10I, 63P</td>
<td>103N, 184V, 215F</td>
<td>43,525</td>
</tr>
<tr>
<td>018-007-001</td>
<td>Plasma</td>
<td>30N, 36, 63P</td>
<td>67N, 103N</td>
<td>10,029</td>
</tr>
<tr>
<td>018-027-002</td>
<td>Plasma</td>
<td>35D, 46L, 63P</td>
<td>118I</td>
<td>212,343</td>
</tr>
<tr>
<td>018-027-004</td>
<td>Plasma</td>
<td>63P</td>
<td>103N</td>
<td>156,790</td>
</tr>
<tr>
<td>018-027-006</td>
<td>Plasma</td>
<td>10I</td>
<td>None</td>
<td>118,334</td>
</tr>
<tr>
<td>018-027-008</td>
<td>Plasma</td>
<td>63P</td>
<td>None</td>
<td>6,288</td>
</tr>
<tr>
<td>PE-009-028</td>
<td>IMC</td>
<td>20R, 63P, 77I, 90M</td>
<td>181C, 184V, 215N/S/Y</td>
<td>NAa</td>
</tr>
<tr>
<td>13-021-027</td>
<td>IMC</td>
<td>30N, 90M</td>
<td>181C, 215Y</td>
<td>NA</td>
</tr>
</tbody>
</table>

a Notation designates codon and altered amino acid residue within HIV-1 PR and RT.

Sequence analysis of HIV-1 PR and RT. Sequence analysis of HIV-1 PR and RT reading frames was performed using the TRUGENE HIV-1 Genotyping Kit and OpenGene DNA Sequencing System according to the manufacturer’s recommendations (Visible Genetics, Inc., Toronto, Canada), and the data were analyzed as described elsewhere (9).

Efficacy assessments. Efficacy assessments of the study device included identification of bases, codons, and resistance mutations in a sequence. For the study examining the effects of different anticoagulant media, test sample sequences collected in EDTA, acid citrate dextrose (ACD), or heparin were compared to sequences determined by the investigational system using samples collected in a VACUTAINER plasma preparation tube (PPT) from the same HIV-1-infected subject. Otherwise, the gold standard for the comparison of the test samples derived from HIV-1-infected subjects was the consensus sequence obtained from multiple independent molecular clones created from the each of the plasma-pheresis samples and sequenced using universal primer sets. HIV-1 samples derived from IMC were compared to a gold standard sequence determined by sequencing of the progeny virus RNA generated from the IMC using standard sequencing chemistry and universal primers. For each test sequence, the proportion of agreement of the entire base sequence between the automatically or manually edited test sequence and the gold standard was determined by comparing the two sequences using the partial and exact match methods. For all analyses of base agreement, the “entire base sequence” refers to 920 bases of the sequence. The proportion of agreement was defined as the number of exactly matching bases between the test and gold standard sequences divided by the total number of bases in the gold standard sequence.

For comparison of different RNA extraction methods, analyses were based on intrasample comparisons, in which sequences obtained with each of the five extraction methods were compared to those of predetermined gold standards. Percent agreement of the bases and number and identity of mutations was calculated for the sequences generated from each test sample relative to the gold standard sequence using the following proportion: [(total number of resistance codons) – (number of resistance codons on which the two sequences disagree)] / (total number of resistance codons). The percent agreement of the bases was calculated based on exact matching of the bases.
**RESULTS**

**Effect of anticoagulants on sequence quality.** To determine the effect of different anticoagulants on sequence data quality, blood was collected from 10 HIV-1-infected individuals (018-027-01 to 018-027-10) (Table 1) using plasma collection tubes containing heparin, EDTA, and ACD. Plasma was separated from cellular components and stored in aliquots at −70°C. A reference sample was also collected into a PPT (Becton-Dickinson, South Plainfield, N.J.), which contains EDTA. For each patient, sequence data from samples collected in heparin-, EDTA-, or ACD-containing tubes were compared to the reference sample. The median plasma HIV-1 RNA titer for these samples was 75,066 copies/ml (range, 6,288 to 2,535,030 copies/ml). As expected, no sequence data were obtained from the heparinized samples. In samples tested after storage at −70°C for 30 days the mean percent agreement between the EDTA and ACD samples and the reference samples was ≥99.7% at both the nucleotide and codon level (Table 2). Mean agreement rates for mutation recognition and wild-type recognition were ≥0.97. Similarly high agreement was observed in samples tested after storage for 3 months (Table 2). Rates of agreement with the reference sample were not significantly different for samples collected in EDTA or ACD.

**Comparison of HIV-1 RNA extraction methods.** To determine the effect of HIV-1 RNA extraction method on sequence data quality, plasma samples from subjects VA-MH-001, VA-MH-003, VA-MH-004, VA-MH-007, and VA-MH-009 were each subjected to extraction by five different procedures in two different laboratories as described in Materials and Methods. Two samples had viral loads in the range of >1,000 to <2,000 copies/ml; two samples had viral loads in the range of >15,000 to <45,000 copies/ml, and one sample had a viral load of >350,000 copies/ml. The mean percent agreement of nucleotide bases between the test sequence and the gold standard sequence for the ten samples extracted with each extraction method was ≥98.17% (Table 3). Similarly, when sequences were analyzed at the amino acid level, mean agreement rates of codons between the test sequence and the gold standard sequence were ≥98.07% for each extraction method. No statistically significant differences in mean agreement rates were observed between extraction methods or between the two testing sites. Wild-type and mutant recognition rates ranged from 0.27 to 1.00. Mean mutation recognition agreement rates were ≥0.773, and mean wild-type recognition agreement rates were ≥0.993 for all extraction methods tested and across both testing sites (Table 3). No differences in sequence quality were observed as a function of sample HIV-1 RNA concentration (data not shown).

**Genotyping samples with low concentrations of HIV-1 RNA.** A panel of plasma samples spiked with HIV-1 from an infectious molecular clone (PE-009-028) (Table 1) to yield final concentrations of 0, 30, 60, 125, 250, 500, and 1,000 HIV-1 RNA copies/ml was used to evaluate sequence reliability using extraction methods designed for low-concentration samples. This panel gave inconsistent results—9 of 28 (32%) samples processed by the two testing sites gave evaluable sequences (two samples for which genotypic data could not be obtained had no HIV-1 RNA). Neither laboratory obtained interpretable sequence data from the samples that contained 30 copies/ml, whereas three of four samples with titers of 1,000 copies/ml produced good sequence data. Similar results were obtained whether the QIAGEN Ultra HIV-1 RNA Extraction Method (3-ml sample volume) or the Roche UltraSensitive AMPLICOR HIV-1 MONITOR Test kit (0.5-ml sample volume) was used for RNA extraction from these samples.

**Effect of freezing and thawing.** To determine the effect of freezing and thawing on assay performance, plasma samples that were collected from two HIV-infected subjects (VA-MH-004 and VA-MH-009) (Table 1) through plasmapheresis were stored as a function of sample HIV-1 RNA concentration (data not shown).
subjected to 10 full freeze-thaw cycles over 2 days at one laboratory. Nominal plasma HIV-1 RNA titers of the two samples were 1,200 and 43,000 copies/ml, respectively. The reference sequence for comparison was the exact sequence of bases and the presence of mutations determined by sequencing of multiple independent clones derived from HIV-1 in the plasmapheresis samples. The mean percentage base agreement for each of the 10 freeze-thaw cycles ranged from 98.1 to 98.5%, with no deterioration through 10 freeze-thaw cycles. Presence of bilirubin, hemoglobin, triglycerides, and antiretroviral compounds had no significant effect on test accuracy. Similarly, presence of other viruses including HBV, HTLV-1, and HIV-2 did not interfere with the assay. Results of this evaluation demonstrated that the TRU-
Plasma samples with HIV-1 RNA titers ≤1,000 copies/ml gave inconsistent results. No sequence data were obtained from plasma samples with an HIV-1 titer of 30 copies/ml, and only 50% of samples with HIV-1 titers of 125 to 500 copies/ml generated a sequence result. However, all of the positive results for samples with 60 to 500 HIV-1 RNA copies/ml came from one laboratory, suggesting that operator-specific factors may have played an important role. More precise definition of the utility of different methods for extracting HIV-1 RNA from low-copy number samples for use in the TRUGENE assay requires evaluation at a larger number of test sites. Nevertheless, these data are consistent with the general recommendation that performance of resistance testing be limited to patients with plasma HIV-1 RNA levels ≥1,000 copies/ml (10).

The difficulty in obtaining sequence data from samples with low copy numbers was surprising, as previous studies had shown that sequence data could be generated from patient samples with virus loads as low as 60 copies/ml provided specimens underwent a centrifugation step to concentrate virions prior to RNA extraction (J. Lawrence, R. M. Lloyd, Jr., L. M. Hough, P. M. Feorino, and M. A. Thompson, Abstr. 7th Conf. Retrovir. Opportun. Infect., abstr. 795, 2000; D. R. McClernon, A. L. Matthews, L. Salter, M. Cronin, and M. St. Clair, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. I-1965, 2001). It is possible that the homogenous template present in test samples containing virus derived from infectious molecular clones of HIV-1 disturbs the stoichiometric relationship of the primers to the virus template binding sites. Some of the primers provided in the TRUGENE HIV-1 Genotyping Kit are degenerate; thus, if the ratio of primer with appropriate binding sites to template is too low, the sample will fail to amplify (at the RT-PCR stage) or CLIP (at the sequencing stage). Alternatively, blood plasma-associated HIV-1 may differ from infectious molecular clones of HIV-1 that confer resistance to currently available antiretroviral agents. Reports of the high prevalence of drug-resistant HIV-1 among patients under care for HIV/AIDS in the United States and the increasing transmission of drug-resistant virus among newly infected individuals heighten the need for resistance testing as part of the clinical care of infected individuals (15; D. D. Richman, S. Bozzette, S. Morton, S. Chien, T. Wrin, K. Dawson, and N. Hellmann, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. LB-17, 2001). Results of the studies reported here demonstrate that the TRUGENE HIV-1 Genotyping Kit and OpenGene DNA Sequencing System are well-suited for this purpose.

WORKS CITED


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