A RealTime HIV-1 viral load assay for automated quantitation of HIV-1 RNA in genetically diverse group M subtypes A–H, group O and group N samples

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Abstract

The Abbott RealTime HIV-1 assay is an automated test for monitoring HIV-1 viral load in plasma samples. The assay uses reverse transcription polymerase chain reaction (RT-PCR) technology with homogeneous real-time fluorescent detection. Automated sample preparation is performed on the m2000sp™ instrument where RNA is isolated using magnetic microparticle technology and dispensed to a PCR tray together with the amplification reagents. The PCR tray is then transferred to the Abbott m2000rt™ instrument for amplification and real-time detection.

The assay utilizes two distinct sets of primers and probes for HIV-1 and for internal control (IC). The IC is processed along with each sample to control for sample recovery and inhibition. The HIV-1 primer and probe sequences are targeted to the integrase (IN) region of the polymerase (pol) gene. Due to the selection of a highly conserved target region and a novel, mismatch tolerant probe design, the assay can quantitate HIV-1 group M subtypes A–H, group O, and group N isolates. The assay provides high reproducibility and a wide dynamic range, allowing quantitation from 40 copies to 10 million copies of HIV-1 RNA per milliliter of plasma. HIV-1 RNA concentrations detected with 95% probability were 25 copies/mL with 1.0 mL of plasma, 39 copies/mL with 0.6 mL of plasma, 65 copies/mL with 0.5 mL of plasma, and 119 copies/mL with 0.2 mL of plasma.

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1. Introduction

Quantitative measurement of HIV-1 RNA levels in peripheral blood has contributed significantly to the understanding of the pathogenesis of HIV-1 infection (Ho et al., 1995) and has been shown to be an essential parameter in the prognosis and optimal management of HIV-1 infected individuals (Mellors et al., 1997; Mylonakis et al., 2001). Decisions regarding initiation or changes in antiretroviral therapy are guided by monitoring plasma HIV-1 RNA levels, CD4+ T cell counts and the patient’s clinical condition.

A variety of nucleic acid based diagnostic assays that quantify plasma HIV-1 RNA levels have been developed to monitor disease progression and response to antiviral drug therapy. These assays utilize technologies such as reverse transcription polymerase chain reaction (RT-PCR) amplification, branched-chain DNA (bDNA) signal amplification, and isothermal nucleic acid sequence-based amplification (Collins et al., 1997; Dyer et al., 1999; Johanson et al., 2001; Sun et al., 1998). One important consideration for all primer/probe-based technologies is the impact of HIV-1 sequence heterogeneity (Burns et al., 2002; Osmanov et al., 2002; Tatt et al., 2001). The genetic variation of HIV-1 presents challenges to the design of quantitative assays that measure HIV-1 RNA levels. Due to the genetic diversity of HIV-1 and the continual redistribution of variant strains, there is a need for molecular tests that are capable of tolerating nucleotide mismatches without compromising sensitivity or the accuracy of viral load measurements (Geelen et al., 2003; Swanson et al., 2005).
The advent of highly effective regimens of antiretroviral therapies that lower plasma viral load by many orders of magnitude as well as the general consensus that highly active anti-retroviral therapy (HAART) should aim to suppress HIV-1 replication as completely as possible has driven the need for sensitive viral load assays with large dynamic ranges (US Department of Health and Human Services (DHHS), 2004). The dynamic ranges of existing assays are limited; when results exceed the assay quantitative ranges, repeat testing using sample dilutions or reflex testing with a more sensitive assay is required (Collins et al., 1997; Erali and Hillyard, 1999).

The central role of viral load testing in clinical management of HIV-1 infected patients has increased laboratory workload considerably, making improvements in technology necessary to provide reduced cycle time and higher throughput (Jungkind, 2001; Muller et al., 2004). Specimen preparation remains the most labor-intensive part of the molecular testing process and accounts for the majority of hands-on time. Thus, automated sample preparation systems are highly desirable for routine clinical testing.

The Abbott RealTime™ HIV-1 assay (RealTime HIV-1; Abbott Molecular Inc., Des Plaines, IL) is an automated test for quantification of HIV-1 RNA in human plasma. Samples are processed using the m2000xp™ automated sample preparation system (Abbott Molecular Inc.) followed by RT-PCR amplification and detection by the m2000rt™ instrument (Abbott Molecular Inc.). In this study, the key performance characteristics of the RealTime HIV-1 assay including linearity, sensitivity, specificity, precision, and detection of diverse HIV-1 groups and subtypes were evaluated. Viral load values obtained from clinical specimens were correlated with AMPLICOR HIV-1 MONITOR v1.5 test results.

2. Materials and methods

2.1. Panels and samples

Panel members for the limit of detection (LoD) study were prepared by diluting a viral standard obtained from the virology quality assurance (VQA) Laboratory of the AIDS Clinical Trial Group (Rush-Presbyterian-St. Luke’s Medical Center, Chicago, IL) in HIV-1 negative plasma. The linearity panel was prepared by diluting HIV-1 Armored RNA (HIV-1 RNA sequences encapsulated within an MS2 bacteriophage particle, Ambion Diagnostics, Ambion Inc., Austin, TX) in HIV-1 negative plasma. Precision panel members with high RNA concentrations (panel members 4–7) were prepared by diluting HIV-1 Armored RNA in HIV-1 negative plasma. Precision panel members with low RNA concentrations (panel members 1–3) were prepared by diluting HIV-1 viral stock (Part #227, Lot #4696N, BBI Diagnostics, West Bridgewater, MA) in HIV-1 negative plasma.

HIV-1 negative human samples used for the specificity study were provided by ProMedDx LLC (Norton, MA). HIV-1 positive human plasma samples used for the correlation study were provided by ProMedDx LLC and Impath-BioClinical Partners Inc. (Franklin, MA).

WHO first international HIV-1 RNA genotype reference panel was obtained from the National Institute for Biological Standards and Control (NIBSC Code #01/466, Hertfordshire, United Kingdom). HIV-1 infected plasma samples tested for the subtype/group quantitation study were collected from individual blood donors from December 1998 to September 2004 by (1) Dr. Leopold Zekeng, Laboratoire de Santé Hygiène Mobile, Yaoundé, Cameroon, (2) Dr. Lazare Kaptué, Université de Yaoundé, Yaoundé, Cameroon, (3) Dr. Lutz Gürthler, Loeffler Institute, University of Greifswald, Greifswald, Germany, (4) Dr. Roberto Badaro, Fundação Bahiana de Infectologia, Bahia, Brasil, (5) Dr. Carlos Brites, Universidade Federal da Bahia, Hospital Universitário, Bahia, Brasil, (6) National Blood Centre, Thai Red Cross Society, Bangkok, Thailand, and (7) Dr. Phillip Hay, The Courtyard Clinic, St. George’s Hospital, London, United Kingdom. All specimens were collected per local regulations in the country of origin at the time of collection. Four group O sample isolates were purchased from BBI Diagnostics (West Bridgewater, MA). Specimens were aliquotted and stored at −70°C until testing. Subtype designations were based on sequence/phylogenetic analysis of gag, pol, and env gp41 immunodominant region as previously described (Swanson et al., 2003).

2.2. Primers and probes

The RealTime HIV-1 primers amplify a sequence of 172 nucleotides (nt) in the pol integrase region of the HIV-1 genome (Johanson et al., 2001). A partially double-stranded fluorescent probe design is used for HIV-1 detection (Fig. 1). The HIV-1 probe is labeled with the fluorophore 6-FAM at the 5′ end. A shorter quencher oligonucleotide, labeled with Black Hole Quencher 1 (BHQ1) at the 3′ end, is complementary to the 5′ end of the HIV-1 probe. In the absence of HIV-1 target, the HIV-1 specific probe fluorescence is quenched through hybridization to the quencher oligonucleotide. In the presence of the HIV-1 target sequence, the HIV-1 specific probe preferentially hybridizes to the target sequence, allowing for fluorescence detection.

In the absence of HIV-1 target

![Image](image1)

In the presence of HIV-1 target

![Image](image2)

Fig. 1. Description of the HIV-1 partially double-stranded linear probe. The “F” on the 5′ end of the probe represents the fluorophore FAM on the HIV-1 probe. The “Q” on the 3′ end of the quencher oligo represents the quencher molecule BHQ1.

**The RealTime HIV-1 assay (RealTime HIV-1; Abbott Molecular Inc., Des Plaines, IL) is an automated test for quantification of HIV-1 RNA in human plasma.**
PCR amplification of the internal control (IC) is accomplished with a different set of primers than those used to amplify HIV-1. The IC primers target a sequence of 136 nucleotides that is derived from the hydroxypyruvate reductase (HPR) gene from the pumpkin plant. The IC Probe is a single-stranded DNA oligonucleotide with the fluorophore VIC at the 5′ end and BHQ1 at the 3′ end. In the absence of IC target sequences, the fluorescence emission of the VIC fluorophore is quenched. In the presence of IC target sequences, probe hybridization to complementary sequences separates the fluorophore and the quencher and allows fluorescent detection.

2.3. Controls and calibrators

The RealTime HIV-1 assay calibrators, positive controls, and internal control are Armored RNA sequences encapsulated within an MS2 bacteriophage particle (Pasloske et al., 1998; Ambion Diagnostics, Ambion Inc., Austin, TX). A 324 base sequence containing 196 bases of HIV-1 pol integrase (HXB2) and 128 bases of vector sequence was inserted into an expression plasmid with E. coli phage MS2 packaging sequences and encapsulated within a bacteriophage coat protein (HIV-1 Armored RNA). The RNA is protected from RNase digestion within the bacteriophage-like complexes and is released during the lysis stage of the sample preparation procedure. HIV-1 Armored RNA was diluted to appropriate concentrations in HIV-1 negative human plasma to manufacture calibrators and positive controls. The negative control consists of HIV-1 negative human plasma. A negative control, a low positive control and a high positive control are included in each run.

A 351 base sequence of the pumpkin HPR gene was inserted into an expression plasmid with E. coli phage MS2 packaging sequences for IC Armored RNA production. IC Armored RNA is diluted to the appropriate concentration in HIV-1 negative plasma to manufacture the internal control reagent. The IC is taken through the entire sample preparation procedure along with each calibrator, control, and specimen and serves as a control for sample preparation recovery, sample inhibition and amplification efficiency. The IC threshold cycle (Ct) value is used to assess the validity of results for each sample.

2.4. Sample preparation

Plasma specimens collected using the anticoagulants EDTA (ethylene diamine tetraacetic acid) or ACD (acid citrate dextrose) are centrifuged at 2000 × g for 5 min at room temperature prior to sample preparation on the automated m2000sp instrument. Sample preparation protocols with input volumes of 1.0, 0.6, 0.5, or 0.2 mL can be used with the RealTime HIV-1 assay. Specimens, assay calibrators and controls are loaded onto the instrument and RNA is isolated using magnetic microparticle technology to capture nucleic acids followed by washes to remove unbound components. The bound nucleic acids are eluted and transferred to a 96 deep-well plate. At the completion of sample preparation, the m2000sp creates an amplification master mix consisting of thermostable rTth polymerase enzyme, manganese chloride activation reagent (MnCl₂), and oligonucleotide reagent containing primers, probes and dNTPs. The m2000sp dispenses 50 μL aliquots of the master mix and 50 μL aliquots of the extracted eluates to a 96-well optical reaction plate. The plate is sealed and transferred to the m2000rt for real-time RT-PCR.

2.5. Amplification and detection

The m2000rt instrument is used for amplification and real-time fluorescence detection. Reverse transcription and PCR amplification are achieved by rTth DNA polymerase in the presence of MnCl₂. The following thermal cycling conditions are used: 1 cycle at 59°C for 30 min; 4 cycles at 95°C for 40 s and 46°C for 30 s; 6 cycles at 92°C for 30 s and 60°C for 30 s; 37 cycles at 92°C for 30 s, 56°C for 20 s plus 2 s auto-increments per cycle and 35°C for 40 s. Fluorescence measurements are recorded during the 35°C step of the last 37 cycles.

This amplification and detection system allows for simultaneous detection of both HIV and IC amplified products at each read cycle. The amplification cycle at which fluorescent signal is detected is proportional to the log of the HIV-1 RNA concentration present in the original sample.

2.6. Calibration and results calculation

A calibration curve is generated with two calibrators (targeted to 1000 and 1,000,000 copies/mL) run in triplicate. The lot specific calibration curve slope and intercept are calculated and stored on the instrument. The HIV-1 RNA concentration in each specimen and control is calculated from the stored calibration curve and patient results are automatically reported on the m2000rt workstation. Depending on the HIV-1 RNA concentration in a specimen, one of four types of result is reported: (1) if the RNA concentration is within the dynamic range of the assay, the concentration is reported as copies/mL, log₁₀ copies/mL, International Units/mL (IU/mL), or log₁₀ IU/mL. One copy of HIV-1 RNA corresponds to 1.74 IU as determined by standardization to the WHO First International Standard (Holmes et al., 2001), (2) if the RNA concentration is above the upper limit of quantitation (ULQ), the result is reported as “>ULQ”, (3) if the RNA concentration is measured below the lower limit of detection (LoD, defined as the HIV-1 RNA concentration detected with 95% or greater probability), the result is reported as “<LoD, detected”. For example “<LoD, detected” result with 1.0 mL sample input volume means HIV-1 RNA was detected, but the concentration was less than 40 copies/mL, (4) if RNA is not detected, the result is reported as “target not detected”.

2.7. Comparator viral load assays

AMPLICOR HIV-1 MONITOR® v1.5: The Monitor v1.5 test (Roche Molecular Systems, Branchburg, NJ) was performed according to the manufacturer’s specifications at LabCorp (Research Triangle Park, NC). The target region of this RT-PCR-based test is gag p24 (Sun et al., 1998).
VERSANT® HIV-1 RNA 3.0 assay: The bDNA test (Bayer Diagnostics, Tarrytown, NY) was performed at Hospital Carlos III (Madrid, Spain) according to the manufacturer’s instructions. This assay targets the HIV-1 \textit{pol} gene (Collins et al., 1997).

3. Results

3.1. RealTime HIV-1 amplification plots

A dilution series of Armored HIV-1 RNA ranging from $7.44 \log_{10}$ copies/mL to $1.7 \log_{10}$ copies/mL (27.5 million copies/mL to 50 copies/mL) was tested in replicates of four. Amplification plots for HIV-1 are shown in Fig. 2(A). The amount of HIV-1 amplification product that is present at each amplification cycle is measured on the \textit{m2000rt} instrument. The amplification cycle at which an increase in fluorescent signal is detected by the \textit{m2000rt} is proportional to the log of the starting concentration of HIV-1 RNA present in the original sample.

The amplification plots for IC from the same run are shown in Fig. 2(B). The RealTime HIV-1 assay uses a non-competitive IC format resulting in a constant Ct for IC throughout the entire dynamic range of the assay. A delay in IC Ct is an indication of poor recovery or PCR inhibition and the sample result is invalidated.

![Fig. 2](image)

**Fig. 2.** RealTime HIV-1 assay amplification plots. HIV-1 RNA ranging from $7.44 \log$ copies/mL to $1.7 \log$ copies/mL were tested in replicates of four using the \textit{m2000sp} and \textit{m2000rt} instruments. (A) Amplification plots of the HIV-1 FAM fluorescent signal versus cycle number are shown. (B) Corresponding amplification plots of internal control VIC fluorescent signal versus cycle number are shown.

### Table 1

<table>
<thead>
<tr>
<th>Panels (copies/mL)</th>
<th>Detected (%)</th>
<th>1 mL</th>
<th>0.6 mL</th>
<th>0.5 mL</th>
<th>0.2 mL</th>
</tr>
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<tbody>
<tr>
<td>250</td>
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<td>N/T</td>
<td>N/T</td>
<td>100</td>
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<tr>
<td>200</td>
<td>N/T</td>
<td>N/T</td>
<td>N/T</td>
<td>98</td>
<td></td>
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<tr>
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<td>N/T</td>
<td>N/T</td>
<td>N/T</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>100</td>
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<td>100</td>
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<td>75</td>
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<td></td>
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<tr>
<td>20</td>
<td>88</td>
<td>77</td>
<td>74</td>
<td>N/T</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>68</td>
<td>47</td>
<td>46</td>
<td>N/T</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>53</td>
<td>23</td>
<td>37</td>
<td>N/T</td>
<td></td>
</tr>
<tr>
<td>Concentration detected with probability of 95%</td>
<td>25</td>
<td>39</td>
<td>65</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>95% CI</td>
<td>20–33</td>
<td>33–49</td>
<td>51–88</td>
<td>102–150</td>
<td></td>
</tr>
</tbody>
</table>

Fifty-seven replicates of each panel member were tested; N/T, not tested.

3.2. Assay sensitivity with 1.0, 0.6, 0.5, and 0.2 mL sample preparation procedures

The RealTime HIV-1 assay sensitivity was determined by testing dilutions (ranging from 5 to 250 copies/mL) of a viral standard obtained from the virology quality assurance (VQA) Laboratory of the AIDS Clinical Trial Group. Fifty-seven replicates of each panel member were distributed across 12 assay runs, three amplification lots, and three \textit{m2000sp} and \textit{m2000rt} instrument pairs. The percentage of replicates detected at each concentration is shown in Table 1. By probit analysis (Finney, 1971), the HIV-1 RNA concentration detected with a probability of 95% was determined to be 25 copies/mL (95% CI 20–33) with 1.0 mL sample input, 39 copies/mL (95% CI 33–49) with 0.6 mL sample input, 65 copies/mL (95% CI 51–88) with 0.5 mL sample input, and 119 copies/mL (95% CI 102–150) with 0.2 mL sample input volume (Table 1).

3.3. Assay linearity

The linearity of the assay was determined using a nine-member panel of Armored HIV-1 RNA with concentrations ranging from 14 to 27,500,000 copies/mL ($1.16$ to $7.44 \log_{10}$ copies/mL). Three runs were performed with four replicates of each panel member on each run, for a total of 12 replicates for each panel member. All 12 replicates of the seven highest panel members were detected, while 11 replicates of the 36 copies/mL panel member and 4 replicates of the 14 copies/mL panel member were detected. A total of 99 replicates were included in the analysis, and the results are shown in Fig. 3. The RealTime HIV-1 assay was shown to be linear within the range tested with a correlation coefficient of 0.999, slope of 0.93 and intercept of 0.26.
Fig. 3. RealTime HIV-1 assay linearity. A panel consisting of dilutions of Armored HIV-1 RNA ranging from 14 to 27,500,000 copies/mL (1.16–7.44 log10 copies/mL) was tested. Expected versus observed concentration are shown on the x and y axes, respectively, in log10 copies/mL.

3.4. Assay specificity

The specificity of the RealTime HIV-1 assay was evaluated by testing 187 HIV-1 serologically negative human plasma specimens. The specimens were tested on three m2000 instrument systems with three lots of amplification reagents. HIV-1 RNA was not detected in any of the samples resulting in an observed specificity of 100% (CI 98.05–100%).

The analytical specificity of the assay was further evaluated by testing 70 specimens obtained from individuals diagnosed with autoimmune disorders or characterized as serologically positive for one of the following markers: systemic lupus erythematosus, anti-nuclear antibodies, rheumatoid arthritis, HBV surface antigen, anti-HTLV-I/II, anti-HCV, and anti-HIV-2. Ten specimens from each category were tested and HIV-1 RNA was not detected in any of these specimens. Additionally, purified nucleic acid from 22 different viruses and microorganisms were added to HIV-1 negative samples to assess for cross-reactivity. HIV-1 RNA was not detected in any of these specimens (data not shown).

3.5. Assay precision

Precision of the RealTime HIV-1 assay was evaluated using a seven-member panel ranging in concentration from 1.86 to 6.77 log10 copies/mL. The panel members were tested in replicates of 75 over the course of 15 runs on three instrument systems and using three lots of amplification reagents. The within run standard deviation (S.D.), between run S.D. and inter-assay S.D. (containing within and between run components) are presented in Table 2. The inter-assay S.D. for the lowest level panel member (72 copies/mL) was 0.19 log10 copies/mL and the inter-assay S.D. ranged from 0.04 to 0.08 log10 copies/mL at all other concentrations tested.

3.6. Assay correlation

Specimens from HIV-1 infected patients were tested with the RealTime HIV-1 assay and Monitor v1.5. Of the 141 specimens tested, 73 were from patients on antiretroviral drug therapy. The observed correlation coefficient between RealTime HIV-1 and Monitor v1.5 is 0.958 with a slope of 0.929 and intercept of 0.146 (Fig. 4). None of the samples differed by more than 1 log copies/mL, and 12 samples (8.5%) differed by more than 0.5 log copies/mL.

3.7. HIV-1 group/subtype detection and quantification

Detection of HIV-1 subtypes/groups by RealTime HIV-1 was evaluated by testing the WHO First International HIV-1 RNA Genotype Reference Panel that includes viral isolates representing group M subtypes A–D, F–H, CRF01_AE, group O, and group N. Results were compared to reported values (Holmes et al., 2003) using other viral load assays (Monitor v1.5, NucliSens HIV-1 QT, bDNA, and LCx HIV RNA Quantitative). The data is presented in Table 3A. RealTime HIV-1 and LCx HIV RNA Quantitative assays quantified all of the isolates including group O and group N. Monitor v1.5, bDNA and NucliSens failed to detect the group O isolate. NucliSens did not detect the subtype G or the group N strains and underquantified subtypes F and H by greater than 1.0 log10 copies/mL relative to the other viral load assays.

Additionally, 90 well-characterized specimens representing group M subtypes A–D, F, G, CRF01_AE, CRF02_AG, and group O (10 each) were tested in the RealTime HIV-1 assay and compared to results obtained with Monitor v1.5 and bDNA. The RealTime HIV-1 assay detected all group M subtypes and group O isolates tested (Table 3B). Monitor v1.5 and bDNA assays detected all group M subtypes tested. Relative to RealTime HIV-1, Monitor v1.5 underquantified (>1.0 log10 copies/mL) 6 of the group M samples, and bDNA under quantified 3 of the group M

<table>
<thead>
<tr>
<th>Panel</th>
<th>Mean concentration (copies/mL)</th>
<th>Mean concentration (log10 copies/mL)</th>
<th>Within run S.D.</th>
<th>Between run S.D.</th>
<th>Inter-assay S.D.</th>
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<td>3</td>
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<td>4</td>
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S.D. values expressed as log10 copies/mL.

* Inter-assay contains within-run and between-run components.
Fig. 4. Comparison of RealTime HIV-1 and Monitor v1.5 quantification. Quantitation values for Monitor v1.5 and RealTime HIV-1 are shown on the x and y axes in log_{10} copies/mL.

Table 3A
Quantitation of the WHO First International HIV-1 RNA genotype reference panel

<table>
<thead>
<tr>
<th>HIV group</th>
<th>HIV subtype</th>
<th>Monitor(^a) v1.5</th>
<th>NucliSens(^b)</th>
<th>bDNA v3.0(^c)</th>
<th>LCx HIV(^d)</th>
<th>RealTime HIV-1(^e)</th>
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<tr>
<td>M</td>
<td>A</td>
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<td>3.06</td>
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<td>B</td>
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</table>

Value expressed as log_{10} copies/mL; ND, not detected.

\(^a\) Amplicor HIV-1 Monitor\(^b\) Test, v1.5.
\(^b\) NucliSens\(^c\) HIV-1QT.
\(^c\) VERSANT\(^d\) HIV-1 RNA 3.0 assay.
\(^d\) LCx\(^e\) HIV-1 RNA quantitative assay.
\(^e\) Abbott RealTime HIV-1 assay.
\(^f\) CRF01_AE based on current subtype nomenclature.
\(^g\) It was subsequently shown to be a mixture of two recombinant (GH and AG) viruses.

Table 3B
Detection of clinical specimens with known HIV-1 group/subtype

<table>
<thead>
<tr>
<th>HIV group</th>
<th>HIV subtype</th>
<th>(n)</th>
<th>RealTime HIV-1(^a)</th>
<th>Monitor(^b) v1.5</th>
<th>bDNA v3.0(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>A</td>
<td>10</td>
<td>10</td>
<td>10(1)</td>
<td>10(1)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>10</td>
<td>10</td>
<td>10(0)</td>
<td>10(0)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>10</td>
<td>10</td>
<td>10(0)</td>
<td>10(0)</td>
</tr>
<tr>
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<td>10</td>
<td>10</td>
<td>10(0)</td>
<td>10(0)</td>
</tr>
<tr>
<td></td>
<td>CRF01_AE</td>
<td>10</td>
<td>10</td>
<td>10(0)</td>
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</tr>
<tr>
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<td>F</td>
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<td>10</td>
<td>10(0)</td>
<td>10(0)</td>
</tr>
<tr>
<td></td>
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<td>10</td>
<td>10</td>
<td>10(3)</td>
<td>10(1)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>10</td>
<td>10</td>
<td>10(2)</td>
<td>10(1)</td>
</tr>
<tr>
<td>O</td>
<td></td>
<td>10</td>
<td>10</td>
<td>0(NA)</td>
<td>7(7)</td>
</tr>
</tbody>
</table>

Note. Numbers in parentheses are the number of specimens with quantitation values more than 1 log_{10} copies/mL lower in Monitor v1.5 or bDNA v3.0 relative to RealTime HIV-1.

\(^a\) Abbott RealTime HIV-1 assay.
\(^b\) Amplicor HIV-1 Monitor\(^b\) Test, v1.5.
\(^c\) VERSANT\(^d\) HIV-1 RNA 3.0 assay.
samples. None of the samples were underestimated by more than 1 log copies/mL by the RealTime HIV-1 assay compared to the other two assays. Monitor v1.5 failed to detect all of the group O samples. Seven of the group O samples were detected by bDNA, but the values were underquantified by >1.0 log_{10} copies/mL relative to RealTime HIV-1.

4. Discussion

Quantitation of HIV-1 has become an indispensable tool for the assessment of patient prognosis and for monitoring antiretroviral therapy. Highly effective regimens of antiretroviral therapy are capable of lowering patients’ plasma viral loads by many orders of magnitude (Saag and Kilby, 1999; Hammer et al., 1997). Clinical laboratories routinely monitor viral loads that range over many orders of magnitude. Consequently, quantitative viral load assays need to provide an extended dynamic range and a low limit of detection in order to assess the effectiveness of antiretroviral therapies. Real-time PCR assays characteristically have a broader dynamic range than PCR assays relying on end point determinations (de Mendoza et al., 2005; Stevens et al., 2005; Wong and Medrano, 2005; Yao et al., 2005). For the RealTime HIV-1 assay, the reported dynamic range is from 40 copies/mL to 10 million copies/mL. This dynamic range is considerably larger than that reported for commercial end-point and even some real-time PCR assays (Elbeik et al., 2000; Sun et al., 1998; Yao et al., 2005). The value of the expanded dynamic range was evident in a comparative study between RealTime HIV-1, bDNA and Monitor v1.5 (Swanson et al., 2006). One notable feature of the RealTime HIV-1 assay design is the use of a non-competitive internal control (IC). The IC is introduced into each sample at the beginning of the RNA extraction process and is simultaneously amplified by RT-PCR and thus, serves as a control for sample preparation, amplification efficiency, and inhibition. The IC RNA sequence is unrelated to the HIV-1 target sequence, and the IC is amplified by a set of primers different from the HIV-1 primers. The non-competitive nature of the IC facilitates reliable quantitation of HIV-1 at input concentrations from 40 copies/mL to 10 million copies/mL. The availability of a test with a wide dynamic range eliminates the uncertainty associated with selecting an assay with the appropriate range (i.e., standard test versus ultrasensitive test), and the requirement for repeat testing, and thus resulting in improved turn-around time and cost savings.

Tests for viral load measurement typically involve sample processing to extract nucleic acids from plasma followed by signal or target amplification and detection. Nucleic acid extraction is the most time-consuming and challenging aspect of the test procedure. Manual methods require skilled technical personnel and extended hands-on time. Automation of this step has the potential to reduce assay variability due to operator-associated errors, improve overall laboratory efficiency, and provide increased throughput. The RealTime HIV-1 assay provides automated sample extraction and automated PCR plate assembly on the m2000sp system. The plate is then transferred to the m2000rt, a thermalcycler with real-time detection and automated data analysis. No post-amplification steps are required. The RealTime HIV-1 assay can be used in conjunction with multiple sample preparation protocols utilizing input volumes ranging from 0.2 to 1.0 mL. Smaller input volumes provide the additional flexibility required in clinical settings where patient sample volumes are limited. Based on results obtained in the assay sensitivity studies, the concentration of RNA detected with 95% probability is 25 copies/mL with 1.0 mL, 39 copies/mL with 0.6 mL, 65 copies/mL with 0.5 mL, and 119 copies/mL with 0.2 mL sample volume inputs. The lower limit of reported quantitative results is 40 copies/mL with 1.0 mL and 0.6 mL, 75 copies/mL with 0.5 mL, and 150 copies/mL with 0.2 mL sample input volumes. The upper limit of reported quantitative results is 10 million copies/mL at all input volumes.

Assay reproducibility is crucial for reliable monitoring of plasma viral loads and for assessing whether a change in viral load is significant. A sustained change in viral load of greater than 0.5 log_{10} is recognized as a biologically relevant change in the level of viral replication (Saag et al., 1996). The combined within run and between run standard deviation for the RealTime HIV-1 assay was 0.19 log_{10} copies/mL at the lowest viral RNA panel level tested (72 copies/mL). At all other HIV levels tested, the standard deviation was less than 0.10 log_{10} copies/mL, making the RealTime HIV-1 assay a reliable tool for detecting significant HIV-1 viral load changes.

The accuracy of the RealTime HIV-1 assay was assured by standardizing the assay to a well characterized and quantitated HIV-1 RNA standard stock obtained from the Virology Quality Assurance Laboratory of the AIDS Clinical Trial Group (Yen-Lieberman et al., 1996), and against the World Health Organization (WHO) 1st International Standard for HIV-1 RNA (97/656) (Holmes et al., 2001). In this study, plasma specimens from 141 HIV-1 infected patients were used to compare performance of RealTime HIV-1 and Monitor v1.5. Viral load values were highly correlated between tests (correlation coefficient of 0.958); of the quantified measurements, 91.5% were within 0.5 log_{10} copies/mL, and 100% of measurements were within 1.0 log_{10} copies/mL. Similar results were obtained in comparative studies between RealTime HIV-1, Monitor v1.5, and bDNA assays (Swanson et al., 2006, 2007). This level of agreement between the RealTime HIV-1 assay and Monitor v1.5 is noteworthy, given the recent study by Gueudin et al. (2007) where on a panel of 88 clinical specimens, values for 26% of samples differed by >0.5 log_{10} copies/mL and 12% of samples differed by >1 log_{10} copies/mL between Monitor v1.5 and Roche Cobas TaqMan. In two other studies where RealTime HIV-1 and Taqman were compared, the two assays correlated well (Braun et al., 2007; Schutten et al., 2007).

HIV-1 exhibits considerable genetic variability as a result of the lack of proof-reading ability of the reverse transcriptase, high viral turnover, genomic recombination during replication, and immune and therapeutic selection pressures (Guimarães et al., 2002; Tatt et al., 2001). Three phylogenetic groups of HIV-1 are recognized: M (major), O (outlier), and N (non-M, non-O). Group M viruses, the cause of most HIV-1 infections, are comprised of 9 subtypes and at least 30 circulating recombinant forms (Los Alamos HIV Sequence Data Base [http://hiv-web.lanl.gov]). Clade B is the most common type
in North America, Europe, and Australia (Delwart et al., 2003; Osmanov et al., 2002). However, in the last decade, prevalence of infection with non-B subtype viruses has increased (Akouamba et al., 2005; Alaeus et al., 1997; Brodine et al., 1995; Dietrich et al., 1997; Guimarães et al., 2002; Hirigoyen and Cartwright, 2005; Lot et al., 2004). The high degree of genetic diversity characteristic of HIV-1 has been shown to compromise detection and accuracy of quantification (Geelen et al., 2003; Gueudin et al., 2003; Nkengasong et al., 1998).

The use of real-time PCR technology offers significant advantages over traditional end-point analysis. However, variability within probe sites can have a significant impact on detection and accuracy in quantitation (Gardner et al., 2003; Nye et al., 2005; Whiley and Sloots, 2005). TaqMan and molecular beacon probes, the two most commonly used probe designs in real-time PCR assays, exhibit high sensitivity in detection of single nucleotide polymorphisms, but may not be the best choice for highly polymorphic targets (Bustin, 2000; Wong and Medrano, 2005). Signal generation with TaqMan probes is dependent on the 5′ to 3′ exonuclease activity of the polymerase during primer extension (Bustin, 2000; Wong and Medrano, 2005). Mismatches can influence the hybridization efficiency of TaqMan probes at relatively high primer extension temperatures, resulting in underquantification or failure of detection. The partially double-stranded probe used in the RealTime HIV-1 assay does not depend on the 5′ to 3′ exonuclease activity of the polymerase enzyme to generate fluorescent signal. Moreover, assay conditions were optimized to take advantage of this property of the partially double-stranded linear probe. During the last 37 cycles, after the denaturation and annealing steps, the temperature is lowered to 35 °C for the fluorescence read step. This low temperature allows efficient hybridization of the probe even in the presence of multiple mismatches in the probe binding site. The enhanced tolerance to mismatches enabled by the partially double stranded linear probe was previously shown in a comparative study between RealTime HIV-1, Monitor v1.5, and bDNA assays (Swanson et al., 2006).

The RealTime HIV-1 assay targets a conserved region in the integrase (IN) region of the polymerase (pol) gene. As therapeutic agents targeting integrase become available, it is conceivable that resistance-associated mutations may arise within the target region of the RealTime HIV-1 assay. Thus far, no mutations associated with resistance to experimental or clinical compounds have been identified within the reverse primer and probe sites of RealTime HIV-1. A few potential mutations have been identified in the forward primer binding region, yet none in the critical 3′ end of the primer binding site (Hazuda et al., 2007; Jones et al., 2007; McColl et al., 2007; Pommier et al., 2005). The RealTime HIV-1 assay has been designed to tolerate nucleotide mismatches. In fact, no discernible impact on performance of the assay was observed when samples containing up to four mismatches in the forward primer were tested (data not shown).

To assess whether reliability of quantitation was influenced by HIV-1 genetic heterogeneity, performance of the RealTime HIV-1 assay was evaluated on two sets of genetically divergent strains. Results obtained with the WHO subtype panel demonstrated that RealTime HIV-1 quantified all of the virus strains, including the group N and O specimens. In contrast, Monitor v1.5, bDNA v3.0 and NucliSens failed to detect the group O strain. Additionally, NucliSens failed to detect the subtype G and group N isolates (Holmes et al., 2003). Performance of RealTime HIV-1 assay was also evaluated on a 90-member panel containing plasma and virus isolates of known HIV-1 group/subtypes. Results were compared with measurements obtained with bDNA v3.0 and Monitor v1.5. For the group M samples, viral loads were highly correlated between tests. Relative to RealTime HIV-1, 93% and 96% of values were within 1.0 log10 for Monitor v1.5 and bDNA v3.0, respectively. Notably, Monitor v1.5 failed to detect all group O samples. Of the 10 group O strains tested, bDNA v3.0 detected 7 but underquantified all 7 samples by 2 log10 copies/mL relative to RealTime HIV-1. Similar results were obtained in a comparative study between RealTime HIV-1, Monitor v1.5, and bDNA assays using serial dilutions of known HIV-1 subtypes (Swanson et al., 2007). Furthermore, in a direct comparison of RealTime HIV-1 and Taqman on a panel of 29 group M samples (Gueudin et al., 2007), RealTime HIV-1 detected all subtypes whereas Taqman failed to detect one subtype G and one subtype H sample. All eight group O samples were quantified by RealTime HIV-1 but were not detected by the Taqman assay. Superior detection and quantitation of diverse HIV-1 strains by the RealTime HIV-1 assay can be attributed to the design of the assay. The assay targets a highly conserved region of the genome, and reagents, cycling conditions, and probe design have been optimized for mismatch tolerance.

In conclusion, the Abbott RealTime HIV-1 is a highly sensitive, reproducible assay with a broad dynamic range for monitoring HIV-1 viral load in HIV-1 infected patients. The automated m2000 system enhances laboratory throughput, reduces hands-on time and operator associated errors, while providing reliable quantitation of genetically diverse strains.

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References


