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# Performance of the automated Abbott RealTi*me*™ HIV-1 assay on a genetically diverse panel of specimens from Brazil

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**Abstract**

The combination of automated sample preparation and real-time RT-PCR for measurement of HIV-1 viral load has the potential to significantly enhance throughput, reduce operator-associated error, and increase assay sensitivity and dynamic range. In this study, RNA was extracted from the plasma of 91 HIV-1 seropositive Brazilian blood donors using the Abbott *m*2000*sp*™ automated sample preparation system. Viral loads measured using the RealTi*me*™ HIV-1 (RealTi*me* HIV-1) assay and the Abbott *m*2000*rt*™ instrument were compared to values obtained in the LCx® HIV RNA quantitative assay. Subtype was determined for 89 of 91 specimens by sequence/phylogenetic analysis of three genomic regions: *gag* p24, *pol* integrase, and *env* gp41. The panel included 69 subtype B, 1 C, 2 F, and 17 recombinant strains. Eighty-seven specimens were quantified by both assays. Two specimens were quantified only in RealTi*me* HIV-1. Two additional specimens below the detection limit of both assays were also negative on PCR amplification. Viral load results were highly correlated, and good agreement was observed between assays with 90% of values within 0.5 $\log_{10}$ copies/ml. The RealTi*me* HIV-1 assay and *m*2000 system offer the advantages of automation while providing reliable quantification of diverse HIV strains.


*Keywords:* Real-time PCR; HIV quantitation; Genetic diversity

## 1. Introduction

Quantitative measurement of human immunodeficiency virus type 1 (HIV-1) in plasma is routinely used to monitor disease progression in untreated infected individuals and to evaluate response to anti-retroviral drug therapy. A variety of technologies used to measure HIV-1 RNA concentrations have been commercialized, including reverse transcription polymerase chain reaction (RT-PCR) amplification, isothermal nucleic acid sequence-based amplification (NASBA), and branched-chain DNA signal amplification (bDNA) (Collins et al., 1997; Dyer et al., 1999; Johanson et al., 2001; Sun et al., 1998). These traditional target amplification-based assays measure the amount of product generated after a fixed number of PCR cycles (end-point determination).

Recently, real-time RT-PCR assays have been developed that continuously monitor the accumulation of product as it occurs (Bustin, 2000; Wong and Medrano, 2005). Real-time PCR is amenable to automation and has several other potential advantages over traditional end-point assays, including increased dynamic range, speed, simplicity, and reduced risk of contamination (Wong and Medrano, 2005). Several recent studies have examined the utility of real-time RT-PCR technology for quantification of HIV-1 RNA (de Mendoza et al., 2005; Rouet et al., 2005; Stevens et al., 2005; Yao et al., 2005). Application of automation to the labor-intensive steps of sample preparation

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and reagent dispensing would significantly enhance throughput and reduce operator-associated variation.

One important consideration for all primer/probe-based technologies is the impact of HIV-1 sequence heterogeneity. The continued evolution and redistribution of HIV presents an ongoing challenge (Los Alamos HIV Sequence Data Base [http://www.hiv-web.lanl.gov]; Osmanov et al., 2002). Moreover, the inherent propensity of HIV-1 to recombine results in a significant increase in overall genetic diversity. In a recent global analysis, recombinant strains accounted for more than 18% of HIV-1 infections (Osmanov et al., 2002). In Europe, North America and South America, where subtype B strains predominate, increasing numbers of non-subtype B and recombinant viruses are being identified (Akouamba et al., 2005; Brindeiro et al., 2003; De Sa Filho et al., 2005; Guimarães et al., 2002; Hirigoyen and Cartwright, 2005; Soares et al., 2003a, 2003b). Early results from a comprehensive surveillance program initiated in France revealed that non-B subtypes accounted for 45% of HIV infections in 2003 (Lot et al., 2004).

Numerous studies have shown that HIV diversity can influence reliability of detection and quantitation (Barlow et al., 1997; Swanson et al., 2005). Inaccurate viral load measurements can have dire clinical consequences (Geelen et al., 2003). Given the importance of monitoring viral load during the clinical management of HIV-1 infected patients, it is essential that assays reliably and accurately quantify genetically diverse viral strains.

In the present study, the Abbott RealTi*m*e™ HIV-1 assay (RealTi*m*e HIV-1; not available in the U.S.) was evaluated using a panel of 91 HIV-1 seropositive plasma specimens from Brazilian blood donors. Performance was compared to the LCx® HIV RNA quantitative assay (LCx HIV; not available in the U.S.), a platform previously demonstrated to reliably quantify diverse HIV strains (de Mendoza et al., 2002; Plantier et al., 2003; Swanson et al., 2000; Troonen et al., 2002). In addition, the genetic subtype of the virus present in each specimen was determined and nucleotide conservation within LCx HIV and RealTi*m*e HIV-1 primer and probe binding sites was assessed.

## 2. Materials and methods

### 2.1. Sample collection

HIV-1 seropositive plasma samples were collected from blood donors in Brazil from 2001 to 2003. The anti-coagulant, CPDA-1, was used to facilitate plasma fractionation. Specimens were divided into aliquots and stored at −70 °C until testing. This study was conducted in conformance with National and Local approval from the Institutional Committee on Ethics of Brazil in Research at Professor Edgard Santos University Hospital, Bahia, Brazil.

### 2.2. Molecular characterization of the plasma panel

Three regions of the HIV-1 genome were targeted for sequence analysis: *gag* p24, *pol* integrase (*pol* IN), and *env* gp41 immunodominant region (*env* IDR). Total nucleic acid was extracted either from 200 to 400 μl of plasma using the QIAamp blood kit (Qiagen Inc., Valencia, CA) or from 1 ml of plasma using the Abbott *m*1000™ automated sample preparation system (Tecan Schweiz, Mannedorf, Switzerland). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using an RNA PCR kit (Applied Biosystems, Foster City, CA) or superscript one step RT-PCR for long templates (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Nested PCR amplification was performed using a GeneAmp PCR kit (Applied Biosystems). Conditions for RT-PCR amplification and methods for sequence/phylogenetic analysis have been described previously (Swanson et al., 2003). Primers not previously reported include the following: *gag* RT-PCR with Mgag13F (5′-TAGTATGGGCAAGCAGGGAG) and Mgag17R (5′-GCTAKRTGYCCTTCYTTGCCACA), *gag* nested PCR with Mgag14F (5′-ATAGAKGTAMAAGACACCAARGA) and Mgag16R (5′-CYTAAARTTRCCTYTCTGCATCA), *env* RT-PCR with JH35F (5′-TGARGGACAATTGGAGAARTGA) and Menv24R (5′-TGTCTTGCTCKCCACCTYCTTCTTC), *env* nested PCR with Menv23F (5′-GGTGGAGAGAGAAAAAGAGC) and JH38 (5′-GGTGARTATCCCTKCCTAAC).

### 2.3. Viral load determination

#### 2.3.1. LCx® HIV RNA quantitative assay

The LCx HIV assay (Abbott Laboratories, Abbott Park, IL) was performed at Abbott Laboratories according to the manufacturer's specifications. This competitive RT-PCR assay targets the *pol* IN region of HIV-1 (Johanson et al., 2001). Using the 1 ml sample preparation protocol, the upper (ULQ) and lower (LLQ) limits of quantitation are 1 000 000 (6.0 $\log_{10}$) copies/ml and 50 (1.7 $\log_{10}$) copies/ml, respectively.

#### 2.3.2. Abbott RealTime™ HIV-1 assay

The RealTi*m*e HIV-1 assay (Abbott Molecular Inc., Des Plaines, IL) was performed at Abbott Laboratories according to the manufacturer's specifications. Total nucleic acid was extracted from 1 ml of plasma using the Abbott *m*2000*sp*™ automated sample preparation system (*m*2000*sp*; Abbott Molecular Inc.). The internal control (IC), derived from the hydroxypyruvate reductase gene of the pumpkin plant, *Cucurbita pepo*, is introduced as Armored RNA® into the sample lysis buffer. The IC is processed simultaneously with each sample. RNA is captured by magnetic microparticles, washed to remove unbound sample components, and eluted. The *m*2000*sp* system also automatically prepares the amplification master mix consisting of: HIV-1 oligonucleotide reagent including primers and probes, thermostable rTth polymerase enzyme, and activation reagent. The liquid handling system transfers master mix and extracted nucleic acid samples to 96-well optical reaction plates. Plates are sealed and placed on an Abbott *m*2000*rt*™ instrument (*m*2000*rt*; Abbott Molecular Inc., not available in the U.S.) for reverse transcription, PCR amplification, and detection/quantitation.

The HIV-1 oligonucleotide probe is a partially double-stranded complex. The long strand, labeled with a fluorophore at the 5′ end, is complementary to the HIV target. The opposite

shorter strand, complementary to the 5′ end of the long strand, is labeled with a quencher at its 3′ end. In the absence of HIV-1 target, the fluorescence is quenched. In the presence of the HIV-1 target sequence, the HIV-1 probe preferentially hybridizes to the target sequence allowing fluorescent detection.

The IC probe is a single-stranded DNA oligonucleotide with a fluorophore at the 5′ end and a quencher at the 3′ end. IC probe fluorescence is quenched in the absence of IC target sequences. In the presence of IC target sequences, IC probe hybridization to complementary sequences separates the fluorophore and quencher and allows fluorescent emission and detection.

The HIV-1 and IC specific probes are each labeled with a different fluorophore, thus allowing for simultaneous detection of both amplified products at each cycle. The amplification cycle at which a reactive level of fluorescent signal is detected by the *m*2000*rt* instrument is proportional to the log of the HIV-1 RNA concentration present in the original sample. The ULQ and LLQ are 10 000 000 (7.0 $\log_{10}$) copies/ml and 40 copies (1.6 $\log_{10}$) copies/ml for the 1 ml sample preparation protocol, respectively. Assay sensitivity, defined as the HIV-1 RNA concentration detected with 95% probability, was 25 copies/ml.

### 2.4. Analysis of agreement between methods

The Bland Altman method (Bland and Altman, 1986) was used to assess agreement between the two viral load tests. The $\log_{10}$ difference of RealTi*m*e HIV-1 minus LCx HIV was plotted against the mean $\log_{10}$ value of the two test results.

## 3. Results

### 3.1. Genetic composition of the panel

Plasma samples were collected from volunteer blood donors identified as HIV-1 seropositive through routine blood screening. Three regions of the genome (*gag* p24, *pol* IN, *env* IDR) were PCR-amplified and sequenced to assign group and subtype. Specimens for which the subtype designation differed between the genetic regions analyzed were categorized as recombinants. Group/subtype was determined for 89 of the 91 specimens. The panel was composed of the following group M subtypes: 69 B, 1 C, 2 F, and 17 intersubtype recombinant strains. The recombinant category, defined by *gag* p24/*pol* IN/*env* IDR included: 1 B/B/BF, 9 F/B/B, 4 F/BF/B, 1 F/BF/F, 1 F/F/B and 1 F/F/U containing an unclassified *env* IDR sequence distinct from known subtypes. Since independent regions of the genome were characterized, it is possible that some strains categorized as recombinants may be dual infections. Subtype could not be determined for two panel members because they were PCR-negative for all three regions examined.

### 3.2. Viral load determination

The RealTi*m*e HIV-1 and LCx HIV assays quantified viral RNA in 89 and 87 of the 91 specimens, respectively (Fig. 1). Viral load measurements ranged from 1.83 to 5.73 $\log_{10}$ copies/ml for RealTi*m*e HIV-1 and from 2.25 to

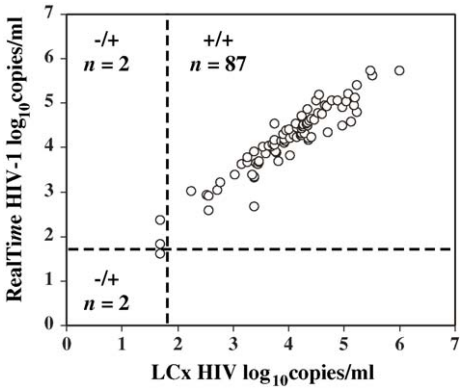


Fig. 1. Comparison of specimen quantification between LCx HIV and RealTi*m*e HIV-1 assays. $\text{Log}_{10}$ values are plotted on both the *x* and *y* axes. The number of specimens is indicated in each quadrant of the scattergram. Dotted lines define the LLQ values for each test. Note that the two points in the −/− quadrant are superimposed.

greater than the ULQ of 6.0 $\log_{10}$ copies/ml in LCx HIV. Two subtype B specimens, quantified at 68 and 229 copies/ml in the RealTi*m*e HIV-1 assay, were below the LLQ of 50 copies/ml in LCx HIV. The two panel members that failed subtype analysis were below the LLQ of both tests. Of the 87 samples quantified by both tests, 86 were within the dynamic ranges; one sample with measurable RNA by both tests was >ULQ of 6.0 $\log_{10}$ in LCx HIV. Seventy-seven of the 86 samples (90%) were within 0.5 $\log_{10}$ RNA copies/ml between tests. Two samples were lower in RealTi*m*e HIV-1 relative to LCx HIV by 0.56 and 0.73 $\log_{10}$ copies/ml (one subtype B and one B/F mosaic). Seven samples (8%) were 0.51 to 0.79 $\log_{10}$ lower in LCx HIV relative to RealTi*m*e HIV-1 (five subtype B, one subtype F, one B/F mosaic). One subtype B sample was measured 1.23 $\log_{10}$ copies/ml lower in LCx HIV (5.04 versus 3.81 $\log_{10}$ copies/ml). When retested in both assays, the viral loads were 4.98 and 4.51 $\log_{10}$ copies/ml in RealTi*m*e HIV-1 and LCx HIV, respectively. The initial RealTi*m*e HIV-1 value and the LCx HIV retest result were used in the correlation analysis. For the 86 samples quantified within the dynamic range of both tests, the observed correlation coefficient was 0.922, with a slope of 0.853 and intercept of 0.772.

Agreement between both methods was determined using the Bland Altman model (Bland and Altman, 1986); results are shown in Fig. 2. The mean difference between RealTi*m*e HIV-1 minus LCx HIV for all quantified samples was 0.18 (range of −0.73 to 0.79) with 90% of the samples within the limits of agreement at ±0.5 $\log_{10}$ copies/ml. Nine points were outside the limits of agreement; seven were higher in RealTi*m*e HIV-1 relative to LCx HIV.

### 3.3. Nucleotide mismatches at primer/probe sites

Nucleotide conservation at the primer and probe binding sites within the *pol* IN target region was examined for both assays. To take into account differences in the lengths of the primers and probes between assays, nucleotide mismatches at each primer or probe site were assessed by calculating a ratio of the number of

Table 1
Nucleotide mismatch ratios at primer/probe binding sites for LCx HIV and RealTi*m*e HIV-1 assays[a]

| | HIV-1 subtype (*n*)[b] | | | | |
|---|---|---|---|---|---|
| | B (74) | C (1) | F (4) | BF (5) | Overall |
| LCx HIV | | | | | |
| Forward | 0.01 (0.00–0.03) | 0.03 (0.03) | 0.03 (0.03) | 0.01 (0.00–0.03) | 0.01 (0.00–0.03) |
| Probe | 0.01 (0.00–0.11) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.01 (0.00–0.11) |
| Reverse | 0.02 (0.00–0.09) | 0.05 (0.05) | 0.03 (0.00–0.05) | 0.03 (0.00–0.05) | 0.02 (0.00–0.09) |
| Total | 0.01 (0.00–0.04) | 0.03 (0.03) | 0.03 (0.01–0.03) | 0.01 (0.00–0.01) | 0.01 (0.00–0.04) |
| RealTi*m*e HIV-1 | | | | | |
| Forward | 0.01 (0.00–0.03) | 0.03 (0.03) | 0.03 (0.03) | 0.01 (0.00–0.03) | 0.01 (0.00–0.03) |
| Probe | 0.05 (0.02–0.12) | 0.02 (0.02) | 0.03 (0.02–0.05) | 0.04 (0.02–0.05) | 0.05 (0.02–0.12) |
| Reverse | 0.02 (0.00–0.12) | 0.04 (0.04) | 0.03 (0.00–0.04) | 0.02 (0.00–0.04) | 0.02 (0.00–0.12) |
| Total | 0.03 (0.01–0.05) | 0.03 (0.03) | 0.03 (0.03–0.04) | 0.02 (0.02–0.03) | 0.03 (0.01–0.05) |

[a] Ratio is calculated as the number of nucleotide mismatches per strain divided by the number of nucleotides per primer or probe site, values shown as mean (range).
[b] Number of specimens.

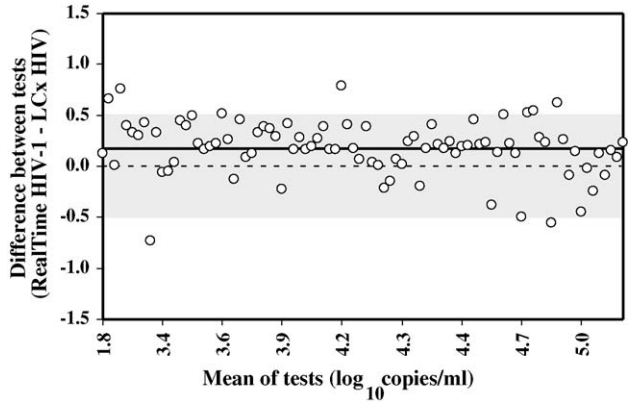


Fig. 2. Agreement between methods. The $\log_{10}$ difference of the RealTi*m*e HIV-1 value minus the LCx HIV value was plotted against the mean $\log_{10}$ value of the two test results. The mean difference between tests is denoted using a solid black line (0.18 $\log_{10}$ copies/ml). Perfect agreement between tests is denoted using a dashed line (0.0 $\log_{10}$ copies/ml). Values within $\pm$0.5 $\log_{10}$ copies/ml are denoted by the gray shaded area.

nucleotide mismatches per HIV strain divided by the number of nucleotides per primer or probe. LCx HIV had a mean ratio of total (primers and probe) nucleotide mismatches of 0.01 (range 0–0.04), whereas RealTi*m*e HIV-1 had a mean of 0.03 (range 0.01–0.05) total mismatches. The forward primer is identical for both tests with a mean number of nucleotide mismatches of 0.01 (range 0–0.03). The reverse primer had a mean of 0.02 (range of 0–0.09 for LCx HIV and 0–0.12 for RealTi*m*e HIV-1). At the probe sites, the mean of nucleotide mismatches was 0.01 (range 0–0.11) for LCx HIV and 0.05 (0.02–0.12) for RealTi*m*e HIV-1. The proportion of nucleotide mismatches that occurs at primer/probe binding sites segregated by group M subtypes B, C, and F and B/F intersubtype mosaic viruses are shown in Table 1.

## 4. Discussion

Sensitive, accurate and reliable quantitation of HIV-1 in plasma is essential for optimal clinical management of HIV-infected patients. Tests for viral load measurement typically involve sample processing to extract nucleic acids from plasma and primer/probe hybridization for target amplification and detection. Nucleic acid extraction is the most time-consuming and challenging aspect of the process. Manual methods require skilled technical personnel and extended "hands-on" time. Therefore, automation of the extraction process should significantly increase reliability and specimen throughput capacity. Application of kinetic or real-time RT-PCR to viral load measurement offers the potential to increase assay dynamic range and reduce the risk of contamination. Given the exceptional level of HIV-1 genetic diversity, viral load assays should target highly conserved regions of the genome and be designed to tolerate nucleotide variations that may occur within primer/probe binding sites.

In the present study, we evaluated performance of the *m*2000 instrument system and the RealTi*m*e HIV-1 assay for quantitation of viral RNA. The automated *m*2000*sp* sample preparation system was used for RNA extraction, RT-PCR master mix preparation, and transfer of master mix and extracted sample into 96-well plates. Plates were manually sealed and transferred to the *m*2000*rt* instrument to measure viral load using the homogeneous format RealTi*m*e HIV-1 assay. Viral load values determined by the RealTi*m*e HIV-1 assay were compared to results obtained with the LCx HIV assay. The LCx HIV assay was previously shown to reliably quantify group M subtypes A–G from various countries including Brazil (Swanson et al., 2000, 2001, 2005).

A 91-member sample panel collected from HIV-1 seropositive blood donors in Brazil was characterized molecularly to determine the genetic composition. Three regions of the genome (*gag* p24, *pol* IN, and *env* IDR) were sequenced for each specimen to assign HIV-1 subtype. Subtype was successfully determined for 89 of the 91 panel members. Based on phylogenetic analysis, the majority (77.5%) of the individuals were infected with subtype B strains, followed by small percentages of subtype C and F viruses (1.1% and 2.2%, respectively). The second largest category (19.1%) was comprised of diverse combinations of B/F intersubtype mosaic genomes. This differs slightly from the distribution shown in a 2003 survey of chronically infected

subjects: 80% subtype B, 5.7% for each of subtype C and F, and 8.6% mosaic strains (Brindeiro et al., 2003). The increased proportion of recombinant viruses observed in the present study may reflect differences in the study population and/or the enhanced capacity to identify mosaic viruses provided by the molecular algorithm for subtype determination that targeted three regions of the genome. Expanded genomic coverage increases the probability of identifying recombinant strains. CRF12_BF strains have previously been identified in Brazil (Brígido et al., 2005). The three regions of the genome used to assess subtype in the present study are subtype F-derived in CRF12_BF, thus not readily distinguishable from subtype F viruses. However, analysis of protease and reverse transcriptase sequences from the two strains designated as subtype F clearly distinguished them from CRF12_BF since they were subtype F-derived and non-recombinant (V.H. and J.H., unpublished observation).

The genetically diverse panel was used to compare performance between the RealTi*m*e HIV-1 and LCx HIV assays. Viral load determinations were highly correlated between tests. For specimens quantified within the dynamic ranges of both tests, the observed correlation coefficient was 0.922. Moreover, there was excellent agreement between tests with 90% of the values within 0.5 $\log_{10}$ copies/ml. Based on initial test results, the viral load values for one specimen differed by >1 $\log_{10}$ copies/ml. To determine whether the difference in quantitation between assays was reproducible, this sample was retested in both assays. Initial and retest values obtained with the RealTi*m*e HIV-1 assay were highly concordant (5.04 and 4.98 $\log_{10}$, respectively), whereas measurements in LCx HIV were more variable (3.81 and 4.51 $\log_{10}$, respectively). The LCx HIV retest value differed by ~0.5 $\log_{10}$ from viral load measurements obtained using RealTi*m*e HIV-1. Inspection of the LCx HIV primer and probe binding sites for this strain revealed no nucleotide mismatches. Thus, there is no apparent molecular basis for the difference in quantification of this specimen by LCx HIV. Two specimens with relatively low viral loads, 68 and 229 copies/ml, respectively, were only quantified by the RealTi*m*e HIV-1 assay. Notably, both specimens were measured near the LLQ of LCx HIV where the coefficient of variation is largest (Johanson et al., 2001). RealTi*m*e HIV-1 is more sensitive than LCx HIV and has less variability in this range. These performance characteristics are likely the basis for the difference observed between assays. Two additional HIV-1 seropositive panel members were below the levels of detection for both tests. If these are in fact HIV-1 infected specimens, they likely have very low viral load set points. Consistent with this interpretation, attempts to amplify all three regions for subtype analysis using a sensitive nested PCR method failed. Although the possibility that these samples were collected from patients receiving antiviral treatment cannot be formally excluded, it seems unlikely since the panel was derived from volunteer blood donors.

The exceptional level of genetic diversity characteristic of HIV-1 makes it a challenging target for detection and quantification. The occurrence of natural polymorphisms within the HIV-1 sequence-specific primer and/or probe binding sites can result in reduced hybridization efficiencies leading to underquantitation or lack of detection (Barlow et al., 1997; Swanson et al., 2005). Although fewer studies have addressed this issue for real-time PCR assays, it is clear that sequence variation in primer or probe binding sites can significantly influence performance (Nye et al., 2005; Whiley and Sloots, 2005). In fact, hydrolysis-type probes (e.g. Taqman) and molecular beacon probes have been shown to have excellent utility for distinguishing between targets that differ by only a single nucleotide (Bustin, 2000; Wong and Medrano, 2005). Implementation of these types of probes for reliable quantification of highly polymorphic targets such as HIV-1 may not prove to be straightforward (Gardner et al., 2003). Early indications suggest that some HIV-1 subtype C strains are underquantified by a commercial assay utilizing molecular beacon probe technology (Gottesman et al., 2005; Stevens et al., 2005). Clearly, to maximize assay reliability (end-point or real-time), it is advantageous to target a region of the HIV-1 genome with a high level of nucleotide conservation. Given the rapid rate of evolution and diversification of HIV-1, it is inevitable that polymorphisms will occur within primer and/or probe binding sites. Thus, it is also critical that assay components and conditions be optimized to tolerate nucleotide mismatches should they occur.

Several previous studies have shown that the primer and probe binding sites of the LCx HIV assay are highly conserved (Swanson et al., 2000, 2001, 2005). This is reflected in the ability of the LCx HIV assay to quantify genetically diverse strains, including group O viruses (de Mendoza et al., 2002; Plantier et al., 2003; Swanson et al., 2005; Troonen et al., 2002). The LCx HIV and RealTi*m*e HIV-1 assays target a similar region within *pol* IN. Since *pol* IN sequence was available as a function of the algorithm used for subtype determination, the level of nucleotide conservation for primer/probe regions could be assessed for both assays. Analysis of the LCx HIV and RealTi*m*e HIV-1 primer binding sites for this panel revealed a high level of nucleotide conservation. For both assays, no mismatches were detected within the forward primer site in 77% of the samples, 0 or 1 in 100% of the panel. No reverse primer nucleotide mismatches were detected in 62% and 60% of samples with LCx HIV and RealTi*m*e HIV-1 assays, respectively. For LCx HIV, 0 or 1 mismatch was present in the reverse primer site in 99% of panel members. The reverse primer used in the RealTi*m*e HIV-1 assay is several nucleotides longer than the primer used in LCx HIV, nevertheless 98% of samples had 0 or 1 mismatch. No mismatches were observed at the critical 3′ end position of any of the primers.

Although primer sequences are quite similar, there are substantial differences between assays with respect to probe design. The RealTi*m*e HIV-1 assay utilizes a partially double-stranded linear probe, a novel class of hybridization probe technology specifically designed to provide the potential for enhanced tolerance to mismatches (K.-C. Luk and J. Hackett, Jr., unpublished observation). The target-specific probe in the RealTi*m*e HIV-1 assay encompasses the highly conserved binding site used in LCx HIV but is nearly two and one-half times the length. As a consequence, the total number of mismatches is higher. For the RealTi*m*e HIV-1 probe, the mean number of mismatches was 2.1 ranging from 1 to 5. The combination of the partially double-stranded probe design and the ability to read signal at

lower temperatures (e.g. 35 °C) has intrinsic advantages over traditional probe designs such as Taqman probes that require hydrolysis at higher temperatures when measuring polymorphic targets (Bustin, 2000; Wong and Medrano, 2005). Reduced stringency resulting from hybridization at lower temperature greatly enhances tolerance to mismatches. The partially double-stranded linear probe technology used in RealTi*m*e HIV-1 shows substantially greater tolerance to mismatches than was observed for the shorter LCx HIV assay probe (Swanson et al., 2005). In fact, the presence of two or three mismatches in the probe site of the RealTi*m*e HIV-1 assay had no discernable impact on quantitation even in circumstances where the LCx probe had no mismatches. This is further illustrated by one unique strain in the panel that contained five nucleotide substitutions in the RealTi*m*e HIV-1 probe region, one in the LCx HIV probe site, and no mismatches at the primer sites for either assay. This virus was quantified at 4.48 $\log_{10}$ copies/ml in RealTi*m*e HIV-1 and at 4.35 $\log_{10}$ copies/ml in LCx HIV. The high level of mismatch tolerance for the partially double-stranded linear probe is consistent with data generated in model systems utilizing transcript templates with engineered mismatches (K.-C. Luk and J. Hackett, Jr., unpublished observation). Thus, RealTi*m*e HIV-1 targets a well-conserved region of HIV-1, and assay components and conditions have been optimized to provide maximal tolerance of genetic variability.

In this study, the RealTi*m*e HIV-1 assay and automated *m*2000 system showed equivalent quantitative performance with LCx HIV on subtype B, F, C, and B/F recombinant strains while providing superior sensitivity and increased dynamic range. Application of automation resulted in increased efficiency and sample throughput. The estimated "hands-on" time for evaluating 48 samples was reduced significantly, from 4.6 h for LCx HIV with manual sample preparation to 0.6 h for RealTi*m*e HIV-1. Moreover, the total time from start of extraction to result was reduced by almost 2 h. The combination of automated RNA extraction and the RealTi*m*e HIV-1 assay offers numerous advantages, including simplicity, homogeneous format for RT-PCR amplification and target detection, reduced contamination risk, enhanced throughput, and increased reliability while providing robust performance on genetically divergent HIV-1 strains.

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