

Quantitative detection of HIV-1 RNA using NucliSens EasyQ HIV-1 assay

Jun Yao^a, Zhen Liu^b, Lung-Sang Ko^{c,*}, Gang Pan^d, Yan Jiang^a

^a National AIDS Reference Laboratory, National Center for AIDS/STD Control and Prevention, Chinese Center for Disease Control and Prevention, No. 27 Nan Wei Road, Xuan Wu District, Beijing 100050, PR China

^b bioMérieux China Ltd., Room 1601-2B&10, East Ocean Centre, No. 24/A Jian Guo Men Wai Street, Chao Yang District, Beijing 100004, PR China

^c bioMérieux China Ltd., 17/F, Yen Sheng Centre, 64 Hoi Yuen Road, Kwun Tong, Kowloon, Hong Kong SAR, PR China

^d Research Centre of Eco-environment sciences, Chinese Academy of Sciences, No. 18 Shuang Qing Road, Beijing 100085, PR China

Received 16 December 2004; received in revised form 30 March 2005; accepted 5 April 2005
Available online 14 June 2005

Abstract

HIV-1 RNA viral load has become the major biological marker for disease prognosis and outcome of antiretroviral therapy in the treatment of HIV-infected individuals. The aim of this study was to compare the performance of the new CE marked NucliSens EasyQ HIV-1 assay with NucliSens HIV-1 QT assay (reference method). NucliSens EasyQ HIV-1 (EasyQ) couples nucleic acid sequence-based amplification (NASBA) with real-time detection using molecular beacons utilizing the NucliSens EasyQ analyzer. NASBA is a sensitive, isothermal, transcription-based amplification system designed specifically for the detection of RNA targets. There was significant correlation ($r=0.878$, $P<0.0001$) between the two different assays in the analysis of clinical samples and the frequency of concordant results (log difference <0.5) was 74%. The two assays detected HIV-1 RNA in 81 specimens, and neither detected (below the lower detection limit, 400 copies/ml for NucliSens HIV-1 QT and 500 IU/ml for EasyQ) HIV-1 RNA in 12 specimens. Three clinical specimens had detectable HIV-1 RNA using the EasyQ only, and two specimens had detectable HIV-1 RNA using the NucliSens HIV-1 QT only. The EasyQ procedure can analyze 48 clinical samples within 6 h. The coefficient of variation of EasyQ ranged from 3.0 to 9.5% (3% at 4.9 log; 5.7% at 3.7 log; 9.5% at 2.7 log). The new assay is shown to be a rapid, convenient, and reliable procedure for HIV-1 RNA viral load monitoring.

© 2005 Elsevier B.V. All rights reserved.

Keywords: NucliSens EasyQ HIV-1; NucliSens HIV-1 QT; NASBA; HIV-1 RNA; Molecular beacons

1. Introduction

HIV-1 and HIV-2 are two related viruses that cause acquired immunodeficiency syndrome (AIDS) in humans. HIV-1 causes the majority of HIV infections worldwide. HIV-1 can be further subdivided into three distinct groups (major, M; new, N; and outlier, O) based on nucleotide sequence differences. Group M can be further divided into at least nine genetically distinct subtypes or clades A–J (Peeters, 2000). In Asia, three major HIV-1 subtypes (B, C and CRF_01AE) are dominant. In China, diverse HIV-1 strains have been identi-

fied. For example, subtype B, C, CRF01_AE and CRF08_BC are circulating in individuals who acquired HIV infection sexually, while subtypes CRF08_BC and CRF07_BC have been detected in intravenous drug abusers. However, in the HIV epidemic among paid blood donors in Henan and Hubei provinces of China, HIV-1 subtype B is the most common (Ruxrungham et al., 2004).

One study examined the fate of 180 homosexual men from whom serial plasma specimens had been collected for more than 10 years (Mellors et al., 1996). In this group, the viral load proved to be a significantly more powerful predictor of long-term survival than the CD4+ cell count, which had been used since the start of the epidemic. Therefore, molecular techniques that measure plasma HIV RNA concentration

* Corresponding author. Tel.: +852 2356 7033; fax: +852 2330 2085.
E-mail address: willis.ko@as.biomerieux.com (L.-S. Ko).

(viral load) are used increasingly for the management of HIV-1 disease.

Three major methodologies have been described for the direct quantification of viral load: (1) branched-chain DNA (bDNA) signal amplification (Pachl et al., 1995) (commercially available as VERSANT[®] HIV-1 RNA 3.0 Assay (bDNA) test, Bayer Diagnostics, Emeryville, CA), (2) reverse transcriptase-polymerase chain reaction (Mulder et al., 1997) (RT-PCR; commercially available as AMPLICOR[™] HIV-1 MONITOR Test version 1.5, Roche Molecular Diagnostics, Pleasanton, CA), and (3) nucleic acid sequence-based amplification (van Gemen et al., 1993) (NASBA; commercially available as NucliSens[™] HIV-1 QT test, bioMérieux Inc., Boxtel, Netherlands). The results of the three assays correlate highly, in addition to being sensitive and accurate (Murphy et al., 2000). However, all three methods are relatively labour intensive and post-amplification steps are required.

NucliSens HIV-1 QT is both FDA approved and CE marked. It performed well in multicenter evaluation compared with other commercially available assays (Ginocchio et al., 2003; Murphy et al., 2000). The NucliSens HIV-1 QT is a NASBA with end-point electro-chemiluminescence (ECL) detection (Deiman et al., 2002). Further refinement has led to the development of the CE marked NucliSens EasyQ HIV-1—a combination of NASBA amplification and real-time detection using molecular beacons utilizing the NucliSens EasyQ analyzer (van Beuningen et al., 2001; Leone et al., 1998).

NASBA is a sensitive, isothermal, transcription-based amplification system specifically designed for the detection of RNA targets (Blank et al., 2002; Collins et al., 2002). It is specific for RNA-based virus in the presence of DNA background. It makes use of the simultaneous enzymatic activities of avian reverse transcriptase, ribonuclease H and bacteriophage T7 RNA polymerase (Compton, 1991; Deiman et al., 2002).

Molecular beacons are hairpin-shaped molecules with an internally quenched fluorophore whose fluorescence is restored upon binding to a target nucleic acid (Tyagi and Kramer, 1996). Molecular beacons have been used in various areas of nucleic acid research, such as single nucleotide polymorphism detection and allele discrimination (Kostrikis et al., 1998). In the EasyQ, there are two different molecular beacons. One molecular beacon, specific for the wild type HIV-1, is labeled with carboxyfluorescein (FAM) while the other molecular beacon, specific for the calibrator, is labeled with carboxy-X-rhodamine (ROX).

Quantification of viral load with the NucliSens EasyQ HIV-1 is determined using a data reduction algorithm in the HIV-1 assay software provided with the detection system hardware (Weusten et al., 2002a, 2002b). In this study, the new EasyQ assay was compared with a reference method (NucliSens HIV-1 QT) using clinical samples and BBI HIV-1 RNA standard panels.

2. Materials and methods

2.1. Study samples

2.1.1. Clinical trial site/clinical samples

The clinical trial was conducted at the National AIDS Reference Laboratory of the National Center for AIDS/STD Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, People's Republic of China. All studies were performed with institutional approval and informed consent was obtained prior to the collection of samples.

Ninety-eight whole-blood samples were determined to be HIV-1 antibody positive using the Vironostika[®] HIV Uni-Form II plus O (bioMérieux, France) assay. The positive diagnosis was confirmed by Western blot analysis (HIV Blot 2.2, Genelabs Diagnostics, Singapore). The samples were obtained from ethnic Chinese donors located in Beijing, Yunnan and Henan provinces of China and were collected by venipuncture into evacuated tubes containing tripotassium ethylenediamine tetra-acetate (Sigma Chemical Co., St. Louis, MO, USA). Tubes were centrifuged (20 min, 1200 × g) and cell-free plasma was removed and immediately frozen in cryogenic vials (Nalge Nunc International, Roskilde, Denmark) at −20 °C, or preferably at −70 °C (recommended by the manufacturer). Plasma samples could be frozen and thawed up to three times without significant loss of HIV-1 RNA (supplier's instruction booklet). Plasma samples were assayed with the NucliSens HIV-1 QT (bioMérieux, France) within two weeks of collection. However, the EasyQ assay was conducted up to one year after sample collection.

2.1.2. HIV-1 RNA panels

To characterize the performance of the EasyQ assay, various RNA test panels were obtained and evaluated.

- (1) BBI HIV-1 RNA Qualification Panel: The BBI HIV-1 RNA Qualification Panel QRD702 (BBI Diagnostics, Boston Biomedica Company, West Bridgewater, MA, USA), is a six-member panel with various concentrations of HIV-1 subtype B RNA (range = <50–8 × 10⁴ copies/ml).
- (2) BBI HIV-1 RNA Clade Performance Panel: The BBI HIV-1 Clade Performance Panel PRD201 (BBI Diagnostics) comprises eight aliquots of HIV-1 virus from tissue culture representing HIV-1 group M clades A–H, inclusive. Each virus culture was quantified by electron microscopy and assigned a genotype by sequencing. The cultures were diluted to approximately 5 × 10⁴ virus particles/ml in defibrinated human plasma negative for HIV-1 RNA. One aliquot of panel diluent was included as a negative control.

2.2. Quantification of HIV-1 RNA

2.2.1. NucliSens HIV-1 QT assay

Nucleic acid isolation, amplification and detection of plasma HIV-1 RNA were performed according to the

manufacturer's instructions provided with each assay kit.

2.2.2. NucliSens EasyQ HIV-1 assay

Nucleic acid isolation was performed as for the HIV-1 QT assay except that EasyQ calibrator was added instead of NucliSens HIV-1 QT calibrator. Amplification and detection were performed by adding 10 μ l of primer solution containing synthetic primers, synthetic molecular beacon probes, nucleotides, dithiothreitol, KCl and MgCl₂ (Refer to package insert) to a tube containing 5 μ l purified nucleic acid extract. The mixture was incubated for 2 min in a NucliSens EasyQ Incubator (bioMérieux, France) at 65 °C and subsequently cooled to 41 °C for 2 min. To each tube, 5 μ l enzyme solution was added. The tubes were sealed with eight-well strip caps (BIOzym TC, Landgraaf, The Netherlands), the reagents mixed and transferred back to the Incubator for 2 min at 41 °C. All tubes were then transferred to a 41 °C temperature controlled NucliSens EasyQ Analyzer (bioMérieux, France). The tubes were read 120 times for fluorophores with an interval of 30 s using a FAM filter set (485/530 nm) and a ROX filter set (590/645 nm).

2.2.3. Quantification method

The NucliSens EasyQ Director Software (bioMérieux, France) quantified the HIV-1 RNA in each sample automatically. Briefly, a curve fit analysis method was used to fit the fluorescent curves. The parameters fitted were fluorescence at time zero, reaction constant and three parameters that describe NASBA amplification and the binding of the molecular beacons. The log of the RNA transcription rate of the sample was compared with the log of the RNA transcription rate of the internal control and to the initial log₁₀ input amount of the internal control. After correction for the batch parameters, the number of HIV-1 copies was calculated. All samples were classified as valid, invalid, positive, or negative based on tolerances of the fit parameter, residual error and minimal fluorescence increase (van Beuningen et al., 2001).

2.3. Determination of clinical sensitivity

The clinical sensitivity of the NucliSens assays was assessed by testing 98 specimens from patients. Samples from these patients might contain variable copy numbers of HIV-1 RNA or no detectable HIV-1 RNA due to successful antiretroviral therapy. Plasma (0.2 ml) from each specimen was evaluated by EasyQ and NucliSens HIV-1 QT. It should be noted that 1 ml is the sample size recommended by the manufacturer.

2.4. Detection and quantification of HIV-1 clades A–H

Each member of the nine-member performance panel (clades A–H and diluent negative control) was tested once with the NucliSens HIV-1 QT and EasyQ.

2.5. Reproducibility and sensitivity studies

According to the supplier's instruction booklet, the limit of detection is 176 copies/ml (NucliSens HIV-1 QT, linear dynamic range from 51 to 5,390,000 copies/ml) and 357 IU/ml (EasyQ, linear dynamic range from 50 to 3,000,000 IU/ml) when the sample input volume is 1 ml. The studies were assessed using BBI HIV-1 RNA Qualification Panel QRD702 (01–06) and BBI HIV-1 RNA Clade Performance PRD201-02 (10-fold serially diluted in RNase-free water). Each sample (0.2 ml) was assayed five times in triplicate by EasyQ.

2.6. Statistical analysis

All data analyses were performed using log₁₀ transformed values. According to the EasyQ supplier's instruction booklet, one copy in NucliSens HIV-1 QT is equivalent to one IU in EasyQ.

Linear regression analysis was used to correlate the linear relationship between the assay values. The correlation coefficient between the NucliSens HIV-1 and EasyQ was determined using SPSS version 10.0 (SPSS Inc., Chicago, IL). The extent of agreement between the two assays was also analyzed (Bland and Altman, 1999).

3. Results

3.1. Clinical sensitivity

Ninety-eight clinical specimens were evaluated with the NucliSens HIV-1 QT and EasyQ assays (Table 1). The limit of detection is 400 copies/ml in NucliSens HIV-1 QT (Bremer et al., 2000) and 500 IU/ml in EasyQ (Table 3). Both assays detected HIV-1 RNA in 81 specimens, and neither detected (below the lower detection limit) HIV-1 RNA in 12 specimens. Three clinical specimens had intermediate HIV-1 RNA copy number (1500, 2100 and 5700 IU/ml, respectively) when assayed with the EasyQ only, and two specimens had low and intermediate HIV-1 RNA copy numbers (170 and 1800 copies/ml, respectively) when assayed with the NucliSens HIV-1 QT only.

The HIV-1 RNA copy number of the 81 positive control samples as measured by EasyQ was plotted against that measured by the NucliSens HIV-1 QT assay (Fig. 1). The cor-

Table 1
Sensitivity of the NucliSens HIV-1 QT and NucliSens EasyQ HIV-1 assays

NucliSens HIV-1 QT	NucliSens EasyQ HIV-1	
	Positive	<LDL ^a
Positive	81	2
<LDL ^a	3	12

^a Below lower detection limit (400 copies/ml in NucliSens HIV-1 QT and 500 IU/ml in NucliSens EasyQ HIV-1).

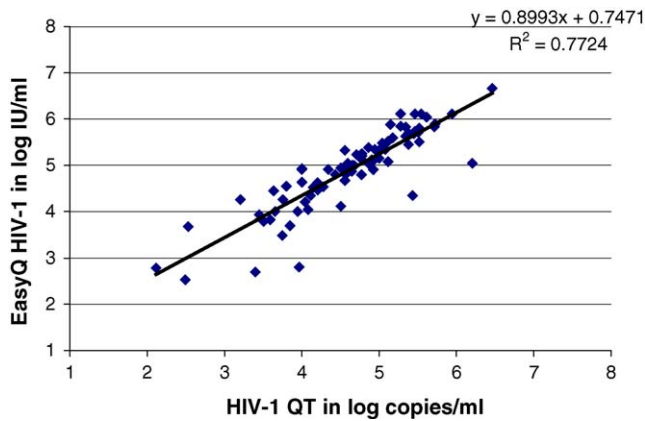


Fig. 1. Concordance of quantitative results obtained by tested with NucliSens EasyQ HIV-1 and NucliSens HIV-1 QT at NCAIDS/STD.

relation coefficient (r) was 0.878, ($P < 0.0001$) indicating a significant correlation between the HIV-1 RNA copy number determined by the two NucliSens assays. From linear regression analysis of the same data, 77.2% of the data points can be represented by the equation of the line ($y = 0.8993x + 0.7471$).

A Bland–Altman plot (Bland and Altman, 1999; Fig. 2) of the data indicated that the mean difference between EasyQ against NucliSens HIV-1 QT was 0.28, while the standard deviation of the mean difference was 0.41 (upper limit to lower limit: 0.54 to -1.1 , with 95% confidence intervals). Sixty-one (75%) of the results were concordant (difference in \log_{10} IU/ml, <0.5) between the two assays. The relatively tight clustering of the data points indicates a high degree of correlation between the two assay methods. The NucliSens HIV-1 QT values were greater than the EasyQ in 76 out of 81 specimens analysed.

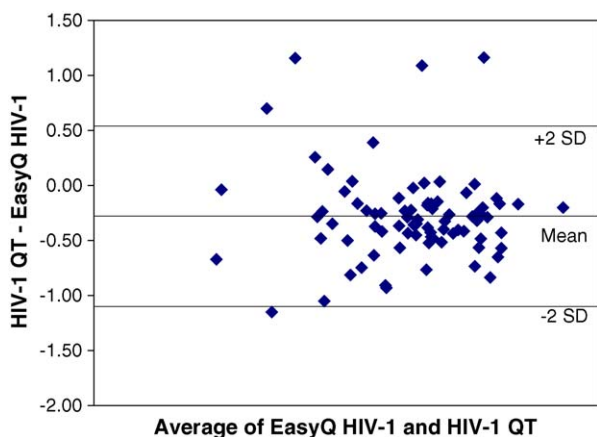


Fig. 2. Comparison between NucliSens EasyQ HIV-1 and NucliSens HIV-1 QT by Bland–Altman plot. The plot displays the differences vs. the means of the same sample measured by two different techniques. Horizontal lines indicate the mean difference plus and minus two times the standard deviation of the differences.

3.2. Detection and quantification of HIV-1 clades A–H

The EasyQ assay was compared with the NucliSens HIV-1 QT assay for the detection and quantification of HIV-1 RNA derived from a panel of samples containing HIV-1 ($\sim 4.7 \log_{10}$ or 5×10^4 virus particles/ml) from group M clades A–H (Table 2). Both assays were able to detect and quantify HIV-1 RNA derived from the eight clades. Overall, the HIV-1 RNA copy numbers obtained with both assays were highly comparable. The difference between the copy numbers detected with the two assays from clade A to clade H was less than 0.5 log. Both assays appeared to underestimate the HIV-1 RNA copy number of subtypes A and C (>1 log than the expected viral particles). Plasma diluent was negative for HIV-1 RNA with both assays.

3.3. Reproducibility and sensitivity studies

Overall precision was determined in a reproducibility study using BBI HIV-1 RNA Qualification Panel QRD702 (01–06) and BBI HIV-1 RNA Clade Performance PRD201-02 (10-fold serially diluted in RNase-free water) with the EasyQ system. The mean assay value was 3.25 ± 0.28 (QRD702-04), 4.18 ± 0.15 (QRD702-05) and 5.06 ± 0.16 (QRD702-06) \log_{10} IU/ml, respectively. QRD702-02 and QRD702-01 were below the lower detection limit ($<LDL$). With QRD702-03, only two out of five separate assays gave quantifiable data (Table 3). With Panel RRD201, the mean assay value was 5.30 ± 0.30 (stock), 4.16 ± 0.34 (1/10 dilution), 3.10 ± 0.30 (1/100 dilution) and $<LDL$ (1/1000 dilution) \log_{10} IU/ml. The coefficient of variation (CV) ranged from 3.0 to 9.5%.

According to the EasyQ manufacturer's specification, the cut-off for HIV-1 RNA quantification is 25 IU/ml (using a 1.0 ml sample input) and the 95% detection rate is 357 IU/ml (1.0 ml input). However, in this study, 0.2 ml input samples were routinely used. Under these conditions, the sensitivity of the EasyQ was 200 IU/ml (QRD702-03) and the 95% detection rate was 500 IU/ml (PRD201-02 1/100 dilution) (Table 3).

4. Discussion

The results from this comparative study show that the clinical sensitivity of the EasyQ assay and the NucliSens HIV-1 QT assay were comparable. Of the 98 clinical samples tested, three samples that had HIV-1 RNA below the limit of detection using the NucliSens HIV-1 QT were positive by EasyQ. In contrast, two samples with HIV-1 RNA below the limit of detection using the EasyQ were positive by NucliSens HIV-1 QT only. Due to the limited sample size available for testing, these five discordant samples were not retested. It is possible that all these samples had low viral loads such that each assay was operating close to its limit of detection (400 copies/ml for NucliSens HIV-1 QT; 500 IU/ml for EasyQ). In addition,

Table 2

Results of BBI HIV-1 RNA Clade Performance Panel PRD201 detected by the NucliSens HIV-1 QT and NucliSens EasyQ HIV-1 assays

Group M genotype ^a	NucliSens EasyQ HIV-1 (log 10 IU/ml)	NucliSens HIV-1 QT (log 10 copies/ml)	Mean difference
Clade A	3.32	2.93	0.39
Clade B	5.05	5.23	-0.19
Clade C	3.67	3.76	-0.09
Clade D	5.28	5.11	0.16
Clade E	4.45	4.81	-0.37
Clade F	4.56	4.85	-0.29
Clade G	5.11	4.87	0.24
Clade H	4.52	4.89	-0.37
Panel diluent	<LDL ^b	<LDL ^b	NA ^c

^a Each positive sample represents a dilution of virus culture to approximately 4.7 log 10 or 5×10^4 virus particles per ml in defibrinated human plasma negative for HIV-1 RNA.

^b Below lower detection limit.

^c Not applicable.

Table 3

NucliSens EasyQ HIV-1 assays precision estimates using BBI HIV-1 RNA Qualification Panel QRD702

HIV-1 RNA member ID ^a	Nominal mean value (log IU/ml)	Results of five separate assays (log IU/ml) ^b					Tested mean value (log IU/ml)	S.D. (log IU/ml) ^c
QRD702-06	4.90	4.79	5.05	5.16	5.16	5.12	5.06	0.16
QRD702-05	4.00	4.10	4.10	4.11	4.14	4.46	4.18	0.15
QRD702-04	3.00	3.42	2.80	3.52	3.34	3.17	3.25	0.28
QRD702-03	2.30	<LDL	<LDL	<LDL	2.98	2.69	NA ^d	NA ^d
QRD702-02	<1.67	<LDL	<LDL	<LDL	<LDL	<LDL	NA ^d	NA ^d
QRD702-01	<1.67	<LDL	<LDL	<LDL	<LDL	<LDL	NA ^d	NA ^d
PRD201-02	4.67	5.10	5.17	5.35	5.08	5.79	5.30	0.30
1/10 dilution ^e	3.67	3.92	3.95	4.24	4.18	4.50	4.16	0.24
1/100 dilution ^e	2.67	2.89	3.00	3.32	2.82	3.50	3.10	0.30
1/1000 dilution ^e	1.67	<LDL	<LDL	<LDL	<LDL	<LDL	NA ^d	NA ^d

^a BBI HIV-1 RNA Qualification Panel QRD702 (06 titer = 4.9 log or 8×10^4 copies/ml; 05 titer = 4 log or 1×10^4 copies/ml; 04 titer = 3 log or 1000 copies/ml; 03 titer = 2.3 log or 200 copies/ml; 02 and 01 titer = <1.67 log or <50 copies/ml) and BBI HIV-1 RNA Clade Performance PRD201-02 (titer = 4.67 log or 50,000 copies/ml).

^b Value of each run is the mean ($n = 3$).

^c S.D. calculated from five runs per sample.

^d Not applicable.

^e Dilutions from the stock virus PRD201-02.

the lower sample input (0.2 ml rather than 1 ml) might lower sensitivity and reproducibility of the two assays. Thus, it is recommended to use the standard input sample. Figs. 1 and 2 demonstrate the high correlation ($r = 0.878$) of the data produced using the two different assay methods. The frequency of concordant results (log difference <0.50) for the 81 clinical specimens in our study was 74% (60/81), which suggests that the assays can be used interchangeably.

Using a commercially prepared panel representative of group M clades A–H (Table 2), both assays provided quantification of viral load to within 0.5 log. The EasyQ assay, in particular, demonstrated reproducible quantification of non-subtype B isolates in contrast to other assays (Hoesley et al., 2002). A precise determination of viral load is very important for the long-term monitoring of patients on anti-retroviral therapy, as it reveals the efficacy of the treatment being administered and may help clinical decision making with respect

to drug selection and dose. Comparing the observed values with the expected values among the different clades (each clade has 4.7 log virus particles as determined by electron microscopy), the observed value of both assays was underestimated (1.0 log) with subtypes A and C. From information supplied by the manufacturer of the BBI clade panel, we averaged the HIV-1 copy number determined by three different detection technologies (VERSANT HIV-1 RNA 3.0 Assay (bDNA) test, NucliSensTM HIV-1 QT test and COBAS AMPLICORTM HIV-1 MONITOR Test version 1.5). The mean HIV-1 RNA copy number for subtypes A and C are 3.41 log and 4.00 log, respectively. Compared with these mean values, both NucliSens assays in this study provided quantification of viral load to within 0.5 log. The problem of underestimation of copy number might be related to some property of the reference samples. Standardization of reference samples for assessment of HIV-1 RNA concentration

has been controversial. The agreement between the viral particle counts and RNA content was poor (Nolte et al., 1998). Therefore, it is suggested that a range of reference samples should be analyzed in conjunction with any assay system used. In Table 3, the coefficient of variation (CV) of EasyQ ranged from 3.0 to 9.5% (3% at 4.9 log; 5.7% at 3.7 log; 9.5% at 2.7 log). As with any molecular assay, the precision increases as the concentration of the analyte increases.

This study showed that the convenience of the EasyQ assay method was superior to that of the NucliSens HIV-1 QT assay method. The EasyQ assay was able to quantify the viral load in 48 clinical samples from nucleic acid extraction to analysis of results within 6 h. In contrast, the same operator using the NucliSens HIV-1 QT assay can handle a maximum of 20–30 samples per day. The increase in sample throughput, together with the rapid and accurate diagnosis, makes the EasyQ method particularly suited for use in routine HIV testing centers.

The NucliSens EasyQ is a closed system comprising a real-time NASBA amplification step with automated data analysis. No post-amplification steps are required. The convenience and decreased workload for laboratory personnel translate directly to cost savings and increased sample throughput. The risk of contamination is decreased with the EasyQ assay method, as the tubes containing the amplification product remain sealed throughout the analysis. With the NucliSens HIV-1 QT assay method, a hybridization step involving the amplicons and probes is required. In the both NucliSens methods, the viral load of each sample is calculated automatically and displayed by the computer. A test report can be printed immediately. Technical staff are not involved in any data manipulation that may decrease laboratory throughput and potentially contribute to error.

During the evaluation, two reactions were invalidated by NucliSens EasyQ software on the NucliSens EasyQ Analyzer because of poor amplification and a discontinuous amplification curve (data not shown). The two samples were retested and acceptable results obtained. In the presence of inhibitors, amplification efficiency will be adversely affected. Under these circumstances, the wild-type RNA (WT) and calibrator (Q) amplification curve will not reach the plateau phase and the result will be invalid. When stringent quality control measures are observed valid results are accurate and reliable.

The linear range of the EasyQ assay is between 50 and 3,000,000 HIV-1 RNA IU/ml (manufacturer's specification). This dynamic range is approximately 0.5 log greater than that of the VERSANT HIV-1 RNA 3.0 Assay (bDNA) test and AMPLICOR™ HIV-1 MONITOR Test version 1.5, respectively (Peter and Sevall, 2004). The ability to quantify HIV-1 RNA over a 5 log span using a single test format is a significant feature of the EasyQ assay, particularly when testing a variety of patient populations with a wide range of viral loads. This is an important asset when a normal clinical laboratory test run includes samples from newly diagnosed untreated patients, patients with histories of multiple drug failures and pediatric patients (Shearer et al., 1997), who generally have

a higher viral load than chronic HIV-1 infected adults. This feature also reduces the number of times incorrect tests are ordered (AMPLICOR™ HIV-1 MONITOR Test version 1.5, standard test versus ultrasensitive test), eliminates the need for reflex repeat testing, improves turnaround time for results and is associated with significant savings in both technical time and reagent costs.

A synthetic HIV-1 RNA internal calibrator provides control over the entire EasyQ procedure from the extraction to detection. Variation in extraction efficiency does not affect the WT to Q ratio prior to amplification. In the presence of amplification inhibitory compounds, WT and Q are affected to the same extent. The use of a synthetic internal calibrator helps prevent false negative assay results. In addition, as no external reference standard curve is required, 48 EasyQ reactions can be used to detect 48 patient samples, increasing the cost-effectiveness of the assay.

In summary, the primer and probe sequences of the CE marked EasyQ assay have been further optimized and the detection technique has been upgraded from an ECL-based end-point detection to real-time detection with molecular beacons. The improved assay gives comparable quantitative results compared with NucliSens HIV-1 QT assays. The EasyQ assay together with the NucliSens EasyQ analyzer offers a rapid, high precision and reliable new system for the real-time detection of HIV-1 RNA.

Acknowledgements

The authors thank bioMérieux for providing the EasyQ assay system for this study. We thank Pei Lijian (Chinese Center for Disease Control and Prevention) for technical assistance and Ron Schoones (bioMérieux) for assistance with statistical analysis. We are grateful to Wim Schippers (bioMérieux) and Paul van del Wiel (bioMérieux) for critical review of the manuscript. We also thank Dr. Richard A Collins for proof-reading the manuscript.

References

- Bland, J.M., Altman, D.G., 1999. Measuring agreement in method comparison studies. *Stat. Methods Med. Res.* 8, 135–160.
- Blank, B.S.N., Meenhorst, P.L., Pauw, W., Mulder, J.W., van Dijk, W.C., Smits, P.H.M., Roelens, F., Middeldorp, J.M., Lange, J.M.A., 2002. Detection of late pp67-mRNA by NASBA in peripheral blood for the diagnosis of human cytomegalovirus disease in AIDS patients. *J. Clin. Virol.* 25, 29–38.
- Bremer, J., Nowicki, M., Beckner, S., Brambilla, D., Cronin, M., Herman, S., Kovacs, A., Reichelderfer, P., 2000. Comparison of two amplification technologies for detection and quantitation of human immunodeficiency virus type 1 RNA in the female genital tract. *J. Clin. Microbiol.* 38, 2665–2669.
- Compton, J., 1991. Nucleic acid sequence-based amplification. *Nature* 350, 91–92.
- Collins, R.A., Ko, L.S., So, K.L., Ellis, T., Lau, L.T., Yu, A.C., 2002. Detection of highly pathogenic and low pathogenic avian influenza

- subtype H5 (Eurasian lineage) using NASBA. *J. Virol. Methods* 103, 213–225.
- Deiman, B., van Aarle, P., Sillekens, P., 2002. Characteristics and applications of nucleic acid sequence-based amplification (NASBA). *Mol. Biotechnol.* 20, 63–179.
- Ginocchio, C.C., Kemper, M., Stellrecht, K.A., Witt, D.J., 2003. Multi-center evaluation of the performance characteristics of the NucliSens HIV-1 QT assay used for quantitation of human immunodeficiency virus type 1 RNA. *J. Clin. Microbiol.* 41, 164–173.
- Hoesley, C.J., Allen, S.A., Raper, J.L., Musonda, R., Niu, Y., Gao, F., Squires, K.E., Aldrovandi, G.M., 2002. Comparative analysis of commercial assay for the detection and quantification of human immunodeficiency virus type 1 (HIV-1) RNA in plasma from patients infected with HIV-1 subtype C. *Clin. Infect. Dis.* 35, 323–325.
- Kostrikis, L.G., Tyagi, S., Mhlanga, M.M., Ho, D.D., Kramer, F.R., 1998. Multicolor molecular beacons for allele discrimination. *Nat. Biotechnol.* 16, 49–53.
- Leone, G., van Schijndel, H., van Gemen, B., Kramer, F.R., Schoen, C.D., 1998. Molecular beacon probes combined with amplification by NASBA enable homogenous, real-time detection of RNA. *Nucleic Acids Res.* 26, 2150–2155.
- Mellors, J.W., Rinaldo, C.R., Gupta, P., White, R.M., Todd, J.A., Kingsley, L.A., 1996. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science* 272, 1167–1170.
- Mulder, J., Resnick, R., Saget, B., 1997. A rapid and simple method for extracting human immunodeficiency virus type 1 RNA from plasma: enhanced sensitivity. *J. Clin. Microbiol.* 35, 1278–1280.
- Murphy, D.G., Cote, L., Fauvel, M., Rene, P., 2000. Multicenter comparison of Roche COBAS AMPLICOR MONITOR Version 1.5. Organ Teknika NucliSens QT with extractor and Bayer Quantiplex Version 3.0 for quantification of human immunodeficiency virus type 1 RNA in plasma. *J. Clin. Microbiol.* 28, 4034–4041.
- Nolte, F.S., Boysza, J., Thurmond, C., Clark, W.S., Lennox, J.L., 1998. Clinical comparison of an enhanced-sensitivity branched-DNA assay and reverse transcriptase-PCR for quantitation of human immunodeficiency virus type 1 RNA in plasma. *J. Clin. Microbiol.* 36, 716–720.
- Pachl, C., Todd, J.A., Kern, G., 1995. Rapid and precise quantification of HIV-1 RNA in plasma using a branched DNA (bDNA) signal amplification assay. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* 8, 446–454.
- Peeters, M., 2000. Recombinant HIV sequences: their role in the global epidemic. Available from www.hiv.lanl.gov/content/hiv-db/immunology/pdf/2000/1/peeters.pdf (accessed August 10, 2004).
- Peter, J.B., Sevall, J.S., 2004. Molecular-based methods for quantifying HIV viral load. *AIDS Patient Care STDs* 18, 75–79.
- Ruxrungtham, K., Brown, T., Phanuphak, P., 2004. HIV/AIDS in Asia. *Lancet* 364, 69–82.
- Shearer, W.T., Quinn, T.C., LaRossa, P., Lew, J.F., Mofenson, L., Almy, S., Rich, K., Handelsman, E., Diaz, C., Pagano, M., Smeriglio, V., Kalish, L.A., 1997. Viral load and disease progression in infants infected with human immunodeficiency virus type 1. Women and Infants Transmission Study Group. *N. Engl. J. Med.* 336, 1337–1342.
- Tyagi, S., Kramer, F.R., 1996. Molecular beacons: probes that fluoresce upon hybridization. *Nat. Biotechnol.* 14, 303–308.
- van Beuningen, R., Marras, S.A., Kramer, F.R., Oosterlaken, T., Weusten, J., Borst, G., van de Wiel, P., 2001. Development of a high throughput detection system for HIV-1 using real-time NASBA based on molecular beacons. In: Raghavachari, R., Tan, W.H. (Eds.), *Genomics and Proteomics Technologies*. Proc. SPIE, vol. 4264, pp. 66–71.
- van Gemen, B., Kievits, T., Schukink, R., van Strijp, D., Malek, L.T., Sooknanan, R., Huisman, H.G., Lens, P., 1993. Quantification of HIV-1 RNA in plasma using NASBA during HIV-1 primary infection. *J. Virol. Methods* 43, 177–188.
- Weusten, J.J., Carpay, W.M., Oosterlaken, T.A., van Zuijlen, M.C., van de Wiel, P.A., 2002a. Principles of quantitation of viral loads using nucleic acid sequence-based amplification in combination with homogeneous detection using molecular beacons. *Nucleic Acids Res.* 30, e26.
- Weusten, J.J., Wouters, P.A., van Zuijlen, M.C., van de Wiel, P.A., 2002b. Stochastic processes defining sensitive and variability of internally calibrated quantitative NASBA-based viral load assays. *Nucleic Acids Res.* 30, e137.