The One-tube Quantitative HIV-1 RNA NASBA: Precision, Accuracy, and Application

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Over the past few years the quantitation of nucleic acids with target sequence amplification methods has been accomplished in many ways. Theoretically the coamplification of internal standard nucleic acid sequences, either DNA or RNA, with the wild-type nucleic acid is superior to other quantitation methods, provided that the amplification efficiencies of wild-type nucleic acid and internal standard nucleic acid are equal.^(1,2) This method has been applied successfully for PCR,⁽³⁾ RT-PCR,^(4,5) and NASBA.⁽⁶⁾

NASBA is an isothermal nucleic acid amplification method that, like 3SR,^(7,8) evolved from TAS,⁽⁹⁾ the first RNA transcription-based amplification method described. Nucleic acid amplification in NASBA is accomplished by the concerted enzyme activities of AMV reverse transcriptase, RNase H, and T7 RNA polymerase, resulting in the accumulation of mainly single-stranded RNA that can readily be used for detection by hybridization methods. The application of an internal RNA standard to NASBA resulted in a quantitative nucleic acid detection method with a dynamic range of four logs but which needed six amplification reactions per quantitation.⁽⁶⁾ Recently, this method was improved dramatically by the application of multiple, distinguishable, internal RNA standards added in different amounts and the electrochemiluminescence (ECL) detection technology.⁽¹⁰⁾ This one-tube quantitative (Q)-NASBA uses only one amplification per quantitation and enables the addition of the internal standards to the clinical sample in a lysis buffer prior to the actual isolation of the nucleic acid.⁽¹⁰⁾ This approach has the advantage that nucleic acid isolation efficiency has no influence on the outcome of the quantitation, which is in contrast to methods in which the internal standards are mixed with the wild-type nucleic acid after its isolation from the clinical sample.

The specific amplification of single-stranded RNA in the presence of double-stranded DNA because of the isothermal nature of NASBA has led to clinical applications in which detection of RNA is essential, such as HIV-1 RNA in viral particles,^(6,10,11) rRNA in *Mycobacteria*,^(12,13) chronic myeloid leukemia (CML) mRNA in blood lymphocytes,⁽¹⁴⁾ and hepatitis C virus, (HCV) RNA in viral particles.⁽¹⁵⁾ The first application of the one-tube Q-NASBA was developed for the quantitation of HIV-1 RNA. However, for clinical diagnostic applications of the one-tube HIV-1 Q-NASBA one should have a good assessment of the precision and the accuracy of the method in the dynamic range that is covered by the internal standards. Only then can firm conclusions concerning changes in HIV-1 viral RNA load, that are not attributable to variation in the quantitative assay itself, be made.

In this study we describe the precision and accuracy of the one-tube HIV-1 Q-NASBA. In this respect, precision is defined as the variation (standard deviation) between quantitative results obtained from the same sample and accuracy is defined as the difference between the quantitative result and the actual input.

This latter objective can be accomplished by the use of a "gold standard" for which an *in vitro* cultured HIV-1 viral stock solution was obtained. In this stock solution the amount of viral particles was determined using electron microscopy.^(11,16,17) Another important application of the gold standard lies in the comparison of quantitative HIV-1 RNA results obtained using different methods at different laboratories. As more and more quantitative HIV-1 RNA detection methods emerge, the widespread use of a gold standard can help to resolve the discrepancies found in viral RNA load at different laboratories.⁽¹⁷⁾

MATERIALS AND METHODS

All one-tube HIV-1 Q-NASBA assays were performed using the NASBA HIV-1 RNA QT kit from Organon Teknika (Turnhout, Belgium), containing all nec-

essary reagents for the nucleic acid isolation, amplification, and detection as described below. A flowchart describing the procedure is shown in Figure 1.

Nucleic acids were extracted from HIV-1 viral particles added to 100 μ l of plasma and 900 μ l of guanidine thiocyanate (GuSCN) lysis buffer. The amount of HIV-1 viral particles was determined using electron microscopy.⁽¹⁶⁾ Before extraction, three RNA internal standards (Q_A , Q_B , and Q_C) from the NASBA HIV-1 RNA QT kit were added to this lysis mixture. The Q_A , Q_B , and Q_C internal standard RNAs only differ from the wild-type RNA and each other by a 20-nucleotide randomized sequence with the same nucleotide composition. This design of internal standards ensures equal efficiency of isolation and amplification. Subsequently 50 μ l of activated silica particle solution was added, binding all nucleic acids (DNA and RNA), in the lysate.⁽¹⁸⁾ The silica particles were washed with GuSCN wash buffer, 70% ethanol, and acetone. After drying, the silica particles, the nucleic acids were eluted in 50 μ l of elution buffer (1 mM Tris at pH 8.5).

Five microliters of the extracted nucleic acids was used as the input for amplification by the addition of 5 μ l of primer mix, followed by a 5 min incubation at 65°C. The reaction was incubated at 41°C, for 5 min, followed by the addition of 5 μ l of enzyme mix and further incubation for 90 min at 41°C. The amount of Q_A , Q_B , Q_C and wild-type amplified products were determined using the ECL detection method⁽¹⁰⁾ in the NASBA QR SYSTEM instrument.

Five microliters of 20-fold diluted amplified products was added to four tubes. Subsequently, 10 μ l of magnetic bead solution with a generic probe for binding all amplified products, 10 μ l of probe solution, specific for Q_A , Q_B , Q_C , and wild type sequences respectively, were added to each tube. Hybridization was carried out at 41°C for 30 min. The tubes were then placed in the NASBA QR SYSTEM ECL detection instrument, and 300 μ l of assay buffer (0.1 M TPA) was added.

The magnetic beads carrying the hybridized products/probe complex are captured on the surface of an electrode by means of a magnet. Voltage applied to this electrode triggers the ECL reaction. The light emitted by the hybridized ruthenium-labeled probes is proportional to the amount of product. Calculations based on the relative amounts of the four products reveal the original amount of wild-type HIV-1 RNA in the sample.

In our laboratory, we have applied physical separation of our NASBA activities (nucleic acid isolation, amplification setup, and detection of amplified products) to prevent carryover contamination. Physical separation was accomplished by the use of different laboratories or the use of fume hoods and flow cabinets. Every designated area is supplied with its own set of laboratory equipment (pipettes), and movement between areas is minimized.

During this study we analyzed 35 negative controls that were placed among positive samples over a period of weeks. Only once did we observe a false-positive result which, upon retesting of the amplified nucleic acid, became negative, indicating a carryover contamination event during the detection setup. During this study we have not observed any cross contamination during nucleic acid isolation or amplification setup.

DYNAMIC RANGE

The dynamic range of the one-tube Q-NASBA is defined by the concentrations of the internal standards (i.e., Q_A , Q_B , and Q_C RNA). Previously,⁽¹⁰⁾ we have shown that accurate quantitation is possible between a factor 10 below the lowest internal standard (Q_C) and a factor 10 above the highest internal standard (Q_A). The amounts used in this study are 10^6 , 10^5 , and 10^4 RNA molecules of Q_A , Q_B , and Q_C , respectively. This enables quantitation of the

FIGURE 1 Flowchart of the experimental procedure of the HIV-1 RNA NASBA QT kit.

wild-type HIV-1 RNA between 10^3 and 10^7 molecules. The dynamic range of the one-tube Q-NASBA was determined using a dilution series of HIV-1 virus in plasma (100- μ l aliquots), containing 1.86, 2.86, 3.86, 4.86, 5.86, and 6.56 log wild-type RNA molecules, respectively. As shown in Figure 2 the quantitation of the lowest wild-type HIV-1 RNA input level (i.e., 1.86 log) revealed an inaccurate result that was not used for linear regression of the data points. The lowest wild-type RNA input level that was quantitated accurately in this study was 2.86 log (725 RNA molecules). In other studies wild-type RNA input levels of 2.6 log (400 RNA molecules) could be quantitated accurately (data not shown).

PRECISION AND ACCURACY

For clinical diagnostic application of quantitative HIV-1 RNA assays it is important to know the precision and accuracy of the assay. Only then can one discriminate between fluctuations in HIV-1 viral load results that are attributable to variation in the assay or to differences in HIV-1 viral load as a consequence of factors such as disease stage or antiviral therapy. In this matter, precision is defined as fluctuations in quantitative results obtained on the same amount of HIV-1 RNA input (i.e., the standard deviation). The accuracy is defined as the difference between the outcome of the quantitation and the actual input of HIV-1 RNA. To determine both, we used three concentrations of the HIV-1 virus stock solution (i.e., the gold standard) as input in 100 μ l of plasma and 900 μ l of lysis buffer and quantitated each concentration 20 times. The results are summarized in Table 1.

The precision was found to increase with increasing wild-type HIV-1 RNA input concentration; precision of 0.23 log was observed when quantitating 3.34 log wild-type HIV-1 RNA input, whereas the quantitation of 6.34 log wild-type HIV-1 RNA input was accomplished with a precision of 0.13 log. These results are in good agreement with the results we obtained previously.⁽¹⁰⁾ The accuracy of the one-tube Q-NASBA ranges from 0.02 to 0.19 log, indicating the correct calibration of the internal standards (Q_A , Q_B , and Q_C RNA) used in the assay.

FIGURE 2 Dynamic range of the one-tube Q-NASBA using 10^6 , 10^5 , and 10^4 RNA molecules of Q_A , Q_B , and Q_C , respectively. The internal standards were mixed with 1.86, 2.86, 3.86, 4.86, 5.86 and 6.56 log wild-type HIV-1 RNA molecules (i.e., dilution series of HIV-1 virus stock solution) in 100 μ l of plasma and 900 μ l of lysis buffer. All quantitations were performed in duplicate. For curve fitting (i.e., linear regression), the wild-type RNA quantitation of 1.86 log was not taken into account.

TABLE 1 Precision and Accuracy of the One-tube Q-NASBA

	Input wild-type HIV-1 RNA molecules (log)		
	3.34	4.86	6.34
Number	19 ^a	20	20
Mean	3.23	4.84	6.15
Precision (= std)	0.23	0.22	0.13
Accuracy	0.11	0.02	0.19

^aOne data point was found to be an outlier by Grubbs' test.

Using a standard deviation of 0.23 (Table 1) for the one-tube Q-NASBA, differences found in HIV-1 viral load that are ≥ 1 log are caused by external factors, like disease stage or antiviral therapy, other than fluctuations in the one-tube Q-NASBA ($P=0.0035$). Differences in the HIV-1 viral load of 0.5 log found as a result of single measurements can be subscribed to external factors with $P=0.13$. However, when differences of 0.5 log are found as a result of duplicate measurements, these can be described to external factors with more certainty ($P=0.03$).

INFLUENCING FACTORS

The one-tube Q-NASBA assay results might be influenced by external factors related to the clinical sample used for analysis. For this reason, the gold standard (input 4.85 log wild-type HIV-1 RNA) was quantitated with addition of 100 μ l of citrate, heparin, or EDTA plasma, or serum. The results are shown in Figure 3. There appears to be no difference in the quantitation of the gold standard in these four sample types. However, the influence of the coagulation process on the quantitation of HIV-1 viral RNA was not investigated in this experiment. Possibly, HIV-1 viral particles are trapped during the coagulation for obtaining serum, resulting in lower viral load results when compared with quantitations performed on plasma samples. To address this problem, comparisons should be made between quantitation of HIV-1-RNA in plasma and serum samples of HIV-1-infected individuals.

FIGURE 3 Wild-type HIV-1 RNA (4.85 log) was quantitated five times in citrate plasma, heparin plasma, EDTA plasma, and serum, respectively. The mean result of the quantitations is given next to each bar.

Three components of plasma and serum that could possibly influence the quantitative outcome of a one-tube Q-NASBA are lipids, hemoglobin, and albumin. These components were added in increasing concentrations to 100 μ l of normal human serum (reference serum) and 900 μ l of lysis buffer in which 4.85 log wild-type HIV-1 RNA was spiked. Neither lipid (3–10%), hemoglobin (40–140 μ M), nor albumin (50–70 g/liter) influenced the outcome of the quantitation (Fig. 4). During the nucleic acid isolation procedure, these compounds are separated from the nucleic acid in such a way that concentrations remaining in the eluate are too low to influence the NASBA amplification.

APPLICATIONS

The advantages that NASBA has over other amplification methods (PCR, LCR, SDA, etc.) for the amplification of RNA has led primarily to the development of NASBA assays for those applications where RNA is a diagnostic target. The determination of HIV-1 viral RNA load is such an application. Both as a research tool and as a diagnostic assay, one can appreciate the value of the one-tube Q-NASBA. The simplicity of the assay, combined with the technical advantages when compared with other quantitative assays, allows quantitative results to be obtained on a large scale. For HIV-1 it is obvious that viral RNA will be an important marker in the research for vaccines and antiviral drugs. In a recent study, the one-tube Q-NASBA was used to assess the efficacy of AZT therapy in asymptomatic HIV-1-infected individuals (S. Jurriaans, pers. comm.). The results of two representative individuals, a responder, patient 8301, and a nonresponder, patient 8306, are depicted in Figure 5. Differences in HIV-1 viral load ≥ 1 log cannot be explained by variation in the one-tube Q-NASBA itself and are therefore explained as effects of the AZT therapy. These results illustrate the useful application of this assay for the rapid assessment of antiviral therapy. In this way, the one-tube Q-NASBA can serve as an important tool in the search for new antiviral drugs.

CONCLUSIONS

A quantitative nucleic acid detection assay is generally composed of three components: nucleic acid extraction, amplification, and detection. The qual-

FIGURE 4 Wild-type HIV-1 RNA (4.85 log) was quantitated in normal human serum (reference serum) with the addition of lipid (3%, 6%, and 10%), hemoglobin (40, 80, and 140 μ M) or albumin (50, 60, and 70 g/liter). The results of the quantitations are stated next to each bar.

FIGURE 5 HIV-1 RNA load in plasma of two asymptomatic HIV-1-infected individuals treated with AZT. The AZT therapy was started at week 0. (○) patient 8301; (△) patient 8306.

ity of the quantitative result is highly dependent on the efficiency and reproducibility of each of the components. In the one-tube Q-NASBA assay the addition of multiple, distinguishable internal standard RNAs to the clinical sample prior to nucleic acid isolation abolishes the effect of nucleic acid isolation efficiency on the quantitation outcome. The ratio of wild-type RNA to internal RNA standards will remain constant independent of the overall extraction efficiency.⁽¹⁰⁾ This ratio determines the quantitative outcome of the assay. Furthermore, because the amplification efficiencies of wild-type and internal standard RNAs are equal, the ratio of these RNAs before amplification will be reflected in the amplification products. This implies that detection of amplified products needs to be absolutely quantitative to determine accurately the ratio of wild-type RNA to internal RNA standards. The ECL detection technology has this property and can measure hybridization signals with a dynamic range of 5 orders of magnitude.^(19,20)

The one-tube Q-NASBA consisting of Boom nucleic acid isolation technology,⁽¹⁸⁾ NASBA amplification,⁽²¹⁾ and ECL detection^(19,20) has a dynamic range of 4×10^2 – 4×10^7 RNA molecules when using 10^6 , 10^5 , and 10^4 RNA molecules of the Q_A , Q_B , and Q_C internal standards, respectively (Fig. 1). Within this dynamic range the precision and accuracy of the assay enables measurement of the changes in HIV-1 RNA load of ≥ 1 log and, very probably, changes of ≥ 0.5 log can be subscribed to factors other than variation in the assay. The use of the Boom nucleic acid isolation method⁽¹⁸⁾ makes the assay suitable for almost any kind of clinical sample (plasma, serum, whole blood, cells). Because of the inclusion of efficient washing steps of the nucleic acids bound to silica, possible NASBA inhibiting factors are removed.

As shown in this study, the concern about contamination that exists for all amplification methods can be dealt with simply by the physical separation of the different NASBA activities (see Materials and Methods). Together with the simplicity of the one-tube Q-NASBA assay, this enables the widespread use of this assay for large-scale quantitation of HIV-1 RNA both in research and clinical settings. With the introduction of a gold standard, enabling comparisons of different quantitation methods, the way will be opened for the application of these assays as a widespread tool in vaccine development and antiviral drug research.

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