

Entirely Automated Quantification of Human Immunodeficiency Virus Type 1 (HIV-1) RNA in Plasma by Using the Ultrasensitive COBAS AMPLICOR HIV-1 Monitor Test and RNA Purification on the MagNA Pure LC Instrument

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The ultrasensitive COBAS AMPLICOR HIV-1 Monitor test was complemented with automated RNA purification on the MagNA Pure LC instrument. This enabled entirely automated ultrasensitive assessment of viral loads in human immunodeficiency virus type 1 (HIV-1)-infected individuals. The detection limit of the fully automated assay and the viral load measurements in 80 clinical samples were found to be in good agreement with those of the conventional ultrasensitive COBAS AMPLICOR HIV-1 Monitor test. The fully automated assay showed markedly reduced hands-on time and was found to be suitable for the routine assessment of HIV-1 viral loads in a clinical diagnostic laboratory.

Quantification of human immunodeficiency virus type 1 (HIV-1)-derived RNA as a measure for viral load in plasma of infected individuals has become indispensable for the management of HIV infections (15, 17). With the introduction of highly active antiretroviral therapy (HAART), viral loads markedly and rapidly decreased in HIV-1-infected individuals (5, 9, 13). This prompted the development of assays with increased sensitivities (2, 4, 14, 16). Growing numbers of HIV-1-infected individuals and the availability of HAART have increased the demand for highly sensitive detection and viral load assessments in routine diagnostic laboratories over recent years. To meet this demand, automation of the various test systems has been initiated (1, 7).

The ultrasensitive COBAS AMPLICOR HIV-1 Monitor (Roche Molecular Systems, Pleasanton, Calif.) is one of the various commercially available assays for the highly sensitive assessment of HIV-1 loads in human specimens. The assay is based on reverse transcription and PCR. According to the manufacturer's documentation quantification is provided from 50 to 75,000 copies/ml (14). This is achieved by concentrating potential HIV particles in plasma samples by applying an ultracentrifugation step prior to RNA purification (2, 16). The assay has been automated with the COBAS AMPLICOR analyzer to the extent of reverse transcription, PCR, and quantification of the amplified products (1, 6). RNA purification from plasma samples is performed manually, which, however, represents the most labor-intensive part of the assay.

To avoid human error and to increase throughput, attempts are being made to completely automate molecular assays for the detection of pathogens in clinical specimens. This also includes the steps of nucleic acid purification, and instruments are being brought to the market to meet this demand (3). The

MagNA Pure LC instrument (Roche Applied Science, Mannheim, Germany) represents such an instrument for automated nucleic acid preparation (3, 8).

The aim of the present study was to complement the ultrasensitive COBAS AMPLICOR HIV-1 Monitor test with automated RNA preparation on the MagNA Pure LC instrument to establish an entirely automated ultrasensitive assay for the assessment of viral loads in HIV-1-infected individuals. The performance of this entirely automated assay was compared with the performance of the conventional ultrasensitive COBAS AMPLICOR HIV-1 Monitor test.

As reference standards for HIV-1, the qualification panel QRD702 (Boston Biomedica, Inc., West Bridgewater, Mass.) was used. For the clinical evaluation of the entirely automated ultrasensitive HIV-1 RNA assay, 81 backup EDTA plasma samples from HIV-infected individuals were used after approval by the local ethics committee. These plasma samples were previously examined with the conventional ultrasensitive COBAS AMPLICOR HIV-1 Monitor test. Plasma from the EDTA blood samples was separated within 30 min and stored at -70°C in aliquots of 1 ml until use.

Throughout the study the AMPLICOR HIV-1 Monitor test, version 1.5, was used. For the conventional procedure 500 μl of plasma was subjected to ultracentrifugation at $23,000 \times g$ for 1 h at 4°C . Then, the supernatant was removed. Lysis buffer (600 μl containing 1.6 μl of internal control RNA [IC]) was added, and RNA was manually extracted from the HIV-1 particles according to the manufacturer's protocol. Reverse transcription, PCR, and quantitative analysis of the PCR products were performed on the COBAS AMPLICOR analyzer according to the manufacturer's instructions.

For the entirely automated approach the RNA preparation was performed on the MagNA Pure LC instrument with software version 2.1 (Roche Applied Science). The MagNA Pure LC contains a section for automated nucleic acid preparation from samples and a postelution section containing a cooling block to keep isolated nucleic acids and reagents until further

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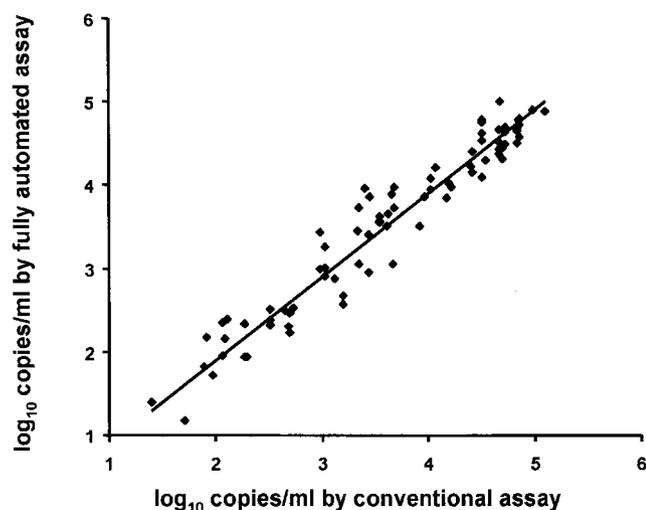


FIG. 1. Correlation between viral load measurements of 80 plasma samples from HIV-1-infected individuals by the fully automated ultrasensitive COBAS AMPLICOR HIV-1 Monitor test and by the conventional ultrasensitive COBAS AMPLICOR HIV-1 Monitor test (regression equation: $y = 1.003x - 0.106$; Spearman rank test: $r = 0.966$, $P < 0.0001$; 95% confidence interval, 0.947 to 0.978).

use. The postelution section can be programmed to perform all steps necessary to prepare PCR mixtures and combine them with the purified nucleic acids. To be compatible with the COBAS AMPLICOR analyzer PCR format, a holder for A rings is placed into the postelution section.

For the automated preparation of HIV-1 RNA, the MagNA Pure total nucleic acid isolation kit, large volume (Roche Applied Science), was used. Its lysis buffer was complemented with the IC of the COBAS AMPLICOR HIV-1 Monitor test. Omitting the ultracentrifugation step used in the manual procedure, 550 μ l of plasma was directly subjected to automated RNA extraction according to the manufacturer's instructions. Elution was performed with 100 μ l of elution buffer. PCR mixtures were prepared in an automated fashion with the reagents of the COBAS AMPLICOR HIV-1 Monitor test. PCR mixtures (50 μ l) and sample RNA (50 μ l) were automatically pipetted into the reaction vessels of the A ring. Thereafter, the A ring was transferred to the COBAS AMPLICOR analyzer for automated reverse transcription, PCR, and quantitation according to the manufacturer's instructions. The low, high, and negative controls provided with the COBAS AMPLICOR HIV-1 Monitor test were always subjected to RNA purification and PCR analysis in both the conventional and the fully automated assays.

In a first step, the required amount of IC per reaction was determined. The IC of the AMPLICOR HIV-1 Monitor test was spiked in graded amounts into the lysis buffer of the MagNA Pure total nucleic acid isolation kit, large volume. Thereafter, automated RNA purification was performed followed by automated PCR analysis. When 1.6 μ l of IC per reaction was used, the optical densities (ODs) specific for the IC amplifications ranged from 0.257 to 0.650 in the 1:9-diluted control tubes. The amount of IC as well as the range of ODs was in agreement with the usage of the conventional ultrasensitive COBAS AMPLICOR HIV-1 Monitor test.

In a second step, a dilution series of a vial of the HIV-1 RNA qualification panel was prepared by using the normal plasma supplied with the COBAS AMPLICOR HIV-1 Monitor test. Then, the detection limit of the fully automated assay was determined. Samples which contained 200, 100, or 50 RNA copies/ml consistently tested positive, eight positive results out of eight tests for each concentration. Samples which contained 25 or 12 or 13 RNA copies/ml were found inconsistently positive, six and three positive results, respectively, out of eight tests each. Thus, the detection limit was found to be 50 RNA copies/ml, which is in complete agreement with the detection limit of the conventional assay as detailed by the manufacturer (14).

The reproducibility of the fully automated assay was determined with 20 low-run and 20 high-run randomly selected controls of the AMPLICOR HIV-1 Monitor test. Thus, inter-assay coefficients of variation of 35.9 and 28.0% were obtained for the low and high controls, respectively. To determine the intra-assay coefficients of variation, 10 replicates of the low and high controls were assessed during one run and were found at 36.0 and 31.8%, respectively.

The entirely automated assay was evaluated in a clinical laboratory setting by assaying viral loads in 81 plasma samples with both the fully automated and the conventional COBAS AMPLICOR HIV-1 Monitor tests. Viral loads in the samples ranged from 25 to 98,000 HIV-1 RNA copies/ml. Figure 1 shows that the viral load measurements with the two assays were in good agreement and were significantly correlated with each other (Spearman rank test; $r = 0.966$; $P < 0.0001$; 95% confidence interval, 0.947 to 0.978). In one sample the IC had failed to be amplified with the fully automated assay. Hence, no result was obtained. Then, the samples of the HIV-1 RNA qualification panel were examined with the fully automated assay. Table 1 shows that the measurements were in good agreement with the expected HIV-1 RNA concentrations of the qualification panel.

The low-, high-, and negative-run controls provided with the COBAS AMPLICOR HIV-1 Monitor test were always tested in parallel with each run and on each A ring. In both the fully automated and the conventional assays, low- and high-run controls were always within the OD range specified in the manufacturer's protocol. The negative-run control always tested negative.

The entirely automated ultrasensitive COBAS AMPLICOR HIV-1 Monitor test was completed after about 7.5 h, when two A rings corresponding to 18 clinical samples were tested. Hands-on time was only 25 min. Automated RNA purification and preparation of PCR mixtures took about 1.5 h, followed by 5.5 h for the run on the COBAS AMPLICOR analyzer. In

TABLE 1. HIV-1 RNA qualification panel QRD702

Panel member	No. of mean RNA copies/ml	No. of RNA copies/ml	
		Examination 1	Examination 2
QRD702-01	<40	<50	<50
QRD702-02	120	140	49
QRD702-03	980	747	651
QRD702-04	5,100	3,300	4,140
QRD702-05	<41,000	<36,200	<24,500

comparison, the conventional ultrasensitive COBAS AMPLICOR HIV-1 Monitor test was completed after about 9.5 h with 1 h of ultracentrifugation, 180 min of hands-on time, and about 5.5 h for the run on the COBAS AMPLICOR analyzer.

Attempts are being made to completely automate molecular assays for the detection of pathogens in clinical specimens. This is thought to produce more reliable and standardized test results (7). Also, due to improved treatment regimens for chronic viral infections, an increasing number of clinical samples have to be analyzed in a routine clinical laboratory. This also applies to the assessment of viral loads in the blood of HIV-1-infected individuals, since the administration of HAART has been found to reduce morbidity and mortality in these patients (11, 12).

To meet the demand for high-throughput testing of low levels of HIV-1 RNA, we have complemented the ultrasensitive COBAS AMPLICOR HIV-1 Monitor test with automated RNA purification on the MagNA Pure LC instrument with the MagNA Pure LC total nucleic acid isolation kit, large volume. As detailed by the manufacturer, this purification protocol is designed to allow the extraction of nucleic acids from 500 up to 1,000 μ l of clinical specimens such as plasma. In using this protocol for the preparation of HIV-derived RNA from plasma, the ultracentrifugation step, which is essential to the conventional ultrasensitive assay, could be omitted. In order to compensate for loss of plasma sample in the liquid handling of the MagNA Pure LC, 550 instead of 500 μ l of plasma was used for the automated HIV-1 RNA preparation. The IC provided with the COBAS AMPLICOR HIV-1 Monitor test seemed to exhibit a similar recovery rate with the automated purification protocol as with the manual purification protocol, since the amount of IC obtained that was suitable for the fully automated assay was also used in the conventional assay. Hence, the amount of IC provided with the COBAS AMPLICOR HIV-1 Monitor test sufficed to perform the same number of fully automated tests as with the conventional assay.

The fully automated ultrasensitive COBAS AMPLICOR HIV-1 Monitor test exhibited a detection limit of 50 HIV-1 RNA copies/ml. When <50 copies/ml were applied to the testing, inconsistent positive or negative results were obtained. This has also been observed with the conventional assay (2, 16).

The fully automated assay exhibited good reproducibility with coefficients of variation of about 30%. This is in accord with those reported previously for the conventional ultrasensitive AMPLICOR HIV-1 Monitor test, i.e., about 30% (2, 16). However, this finding seems to contradict results of a recent evaluation of the MagNA Pure LC instrument, which showed very low intra-assay variances in a combination of nucleic acid purification on the MagNA Pure LC and real-time PCR on the LightCycler instrument (3, 8). The reported low intra-assay variances were most likely due to the highly reproducible preparation of nucleic acids by the MagNA Pure LC, as variations in real-time PCR assays have mainly been attributed to variations of the nucleic acid purification procedures (10). Therefore, the precision of data obtained with the entirely automated ultrasensitive COBAS AMPLICOR HIV-1 Monitor test may mainly reflect the analysis procedure on the COBAS AMPLICOR instrument.

In the evaluation of clinical samples, good agreement of viral

load measurements was obtained when the fully automated assay and the conventional assay were compared (Fig. 1). The regression obtained with both measurement data sets implies that the fully automated assay exhibited a similarly good linearity as the conventional assay, since viral loads obtained with both assays were highly significantly correlated with each other. Also, the testing of the HIV-1 reference panel yielded acceptable results.

In 1 out of 81 samples the IC was not amplified to detectable levels. This sample could not be retested due to lack of material. So, it remains unclear whether the IC was lost during the RNA purification or whether inhibitors of the PCR prevented its amplification. Apart from that, the fully automated assay was found to be robust and suitable for routine clinical application. The low- and high-run controls were always obtained within the specified ranges. Also, the negative control always tested negative, and contaminations during the clinical evaluation of the fully automated assay have not been observed.

The complete automation of the ultrasensitive COBAS AMPLICOR HIV-1 Monitor test was found to be markedly labor saving. Compared to the conventional assay on the COBAS AMPLICOR analyzer, the hands-on time was reduced by approximately 85%. Furthermore, the overall time required for the testing was reduced by 2 h owing to the omission of the ultracentrifugation step and the shorter time required for the automated RNA preparation than for the manual RNA preparation procedure. Automated molecular assays are thought to minimize human error, to minimize contaminations, and to reduce costs of labor. This is usually achieved by a higher degree of instrumentation and increased costs of consumables. In this regard, the automated RNA purification on the MagNA Pure LC increased the overall costs of consumables of the ultrasensitive COBAS AMPLICOR HIV-1 Monitor test by only 8% per patient sample.

In conclusion, the ultrasensitive COBAS AMPLICOR HIV-1 Monitor test was complemented with automated RNA purification on the MagNA Pure LC instrument to arrive at an entirely automated molecular assay for the ultrasensitive assessment of HIV-1 loads in plasma. Compared to the conventional assay on the COBAS AMPLICOR analyzer, the completely automated assay proved to be reliable and markedly labor saving and was found to be suitable for the routine clinical monitoring of HIV-1 viral loads in plasma.

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