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# Evaluation of the Ultrasensitive Roche Amplicor HIV-1 Monitor Assay for Quantitation of Human Immunodeficiency Virus Type 1 RNA

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**The ultrasensitive Amplicor HIV-1 Monitor test (Roche Diagnostic Systems) was evaluated for precision, linearity, and sensitivity and was compared to the standard Amplicor assay. The ultrasensitive assay reliably quantified samples in the range from 50 to 50,000 human immunodeficiency virus type 1 RNA copies/ml with acceptable correlation with the standard Amplicor test.**

Quantitative viral load measurement of human immunodeficiency virus type 1 (HIV-1) has become a valuable tool for the management of HIV-1-infected patients. Levels of HIV-1 RNA in plasma have been shown to be useful as markers of prognosis, for the assessment of disease progression, and for monitoring the efficacy of antiretroviral drug therapy (3, 5, 7). The recent advent of combination drug therapies allows viral load suppression for many patients to a level below the quantitation limit of the currently available HIV-1 RNA assays. New methods for measuring HIV-1 RNA at levels below 400 to 500 HIV-1 RNA copies/ml have been developed, and recent studies have begun to evaluate the clinical significance of viral levels in this range. Early reports suggest that suppression to <50 HIV-1 RNA copies/ml compared to 50 to 400 HIV-1 RNA copies/ml is associated with a more sustained response (2, 6). These reports support the use of ultrasensitive HIV-1 RNA quantitation methods for evaluating and optimizing antiretroviral therapy.

Viral load measurement at extremely low levels is subject to constraints imposed by the statistics of viral particle sampling as well as the inherent variability of the assay technology. A clinically useful ultrasensitive assay will need excellent reproducibility and reliability, with an appropriate sample volume. In order to assess the performance characteristics of the ultrasensitive assay with the Amplicor HIV-1 Monitor test, precision, linearity, and sensitivity were evaluated and comparison with other methods was made.

The ultrasensitive modification of the Amplicor HIV-1 Monitor test was performed according to the protocol provided by Roche Molecular Systems, Inc. (Somerville, N.J.). Specimens were EDTA plasma samples submitted for HIV-1 viral load measurement to ARUP Laboratories, Salt Lake City, Utah. Specimens were drawn in EDTA tubes, and the plasma was removed from the cells within 30 min. The specimens were frozen at ≤−20°C within 2 h of separation and shipped frozen.

Within-run precision was determined with five samples analyzed in replicates of four or five in one run. The samples had low viral loads ranging from 30 to 500 HIV-1 RNA copies/ml. The mean percent coefficient of variation (% CV) for HIV-1 RNA copies per milliliter was 19.4%, and the mean standard deviation of the HIV-1 RNA $\log_{10}$(copies per milliliter) was 0.09. A summary of the within-run precision data is presented in Table 1.

The between-run precision was determined with in-house controls prepared by using clinical specimens diluted in HIV-1-seronegative plasma. Precision was evaluated over 37 separate runs for the low control and over 38 separate runs for the high control. The mean value for the low control was 200 HIV-1 RNA copies/ml, and the mean value for the high control was 17,000 HIV-1 RNA copies/ml. The % CV for HIV-1 RNA copies per milliliter for the low and high controls was 30.8 and 32.2%, respectively. The standard deviation of the HIV-1 RNA $\log_{10}$(copies per milliliter) for the low and high controls was 0.13 and 0.12, respectively. A summary of the between-run precision is presented in Table 1.

Serial dilutions of three clinical specimens were prepared in HIV-1-seronegative plasma, and the HIV-1 RNA levels were measured in the ultrasensitive assay. In order to describe the wide range of values obtained in the dilution series, the data for percent recoveries are presented as the percent observed of total expected for the $\log_{10}$ of the HIV-1 RNA copies per milliliter. The raw data and percent recoveries are presented in Table 2. The range of recoveries was 81%, for the dilution with 40 HIV-1 RNA copies/ml expected, to 105%, for the dilution with 80 HIV-1 RNA copies/ml expected. A plot of the observed values versus the expected values is presented in Fig. 1. The equation for the regression line is $y = 0.958x + 0.054$, $r^2 = 0.994$, $r = 0.997$, for $n = 15$ points. These data show that the ultrasensitive assay is linear over the range from 50 to 50,000 HIV-1 RNA copies/ml.

Viral load levels were determined for 32 clinical samples by both the ultrasensitive assay and the standard Amplicor HIV-1 Monitor test. The range of samples tested was from 170 to 60,000 HIV-1 RNA copies/ml as determined in the ultrasensitive assay and 400 to 50,000 HIV-1 RNA copies/ml as determined in the standard assay. The regression equation for the correlation was $y = 1.088x - 0.343$, $r^2 = 0.904$, $r = 0.951$ for $n = 32$ samples. The reportable range for the ultrasensitive assay is 50 to 50,000 HIV-1 RNA copies/ml and 400 to 750,000 HIV-1 RNA copies/ml for the standard assay. Several samples which were >400 copies/ml in the standard assay measured >50 but <400 copies/ml in the ultrasensitive assay. This variation may be a consequence of the imprecision at low concentrations, since all samples were run in singlet on both assays. As shown in Fig. 2, the correlation is acceptable over the overlapping range of the two assays, 400 to 50,000 HIV-1 RNA copies/ml.

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TABLE 1. Within-run and between-run precision in the ultrasensitive Amplicor HIV-1 Monitor test

| Parameter | Value for sample | | | | | | |
|---|---|---|---|---|---|---|---|
| | Within-run | | | | | Between-run | |
| | A | B | C | D | E | Low | High |
| No. of HIV-1 RNA copies/ml | | | | | | | |
| Mean | 498 | 24 | 222 | 108 | 33 | 226 | 16,866 |
| SD | 120.4 | 70.4 | 36.8 | 26.2 | 1.0 | 69.6 | 5,437 |
| % CV | 24.2 | 29.3 | 16.6 | 24.2 | 2.9 | 30.8 | 32.2 |
| *n* | 4 | 5 | 4 | 4 | 4 | 37 | 38 |
| No. of HIV-1 RNA $\log_{10}$(copies/ml) | | | | | | | |
| Mean | 2.7 | 2.4 | 2.3 | 2.0 | 1.5 | 2.3 | 4.2 |
| SD | 0.11 | 0.14 | 0.07 | 0.12 | 0.01 | 0.13 | 0.12 |
| % CV | 3.9 | 5.9 | 3.2 | 5.8 | 0.8 | 5.7 | 2.9 |
| *n* | 4 | 5 | 4 | 4 | 4 | 37 | 38 |

Clinical specimens below the detection limit (400 HIV-1 RNA copies/ml) of the standard Amplicor HIV-1 Monitor test were evaluated in the ultrasensitive assay. The samples chosen for this study were biased toward a likelihood of being >50 HIV-1 RNA copies/ml. This was done by selecting samples which were not quantifiable but had optical densities (OD) at 450 nm (at a dilution factor of 1 in the assay detection step) which were greater than the expected background of 0.070 OD U, as specified in the Amplicor package insert. The assumption was that these samples with OD readings greater than background may actually contain HIV-1 RNA at levels that are not quantifiable by the standard assay. Ten samples chosen by these criteria were evaluated in the ultrasensitive assay. A summary of the data is presented in Table 3. HIV-1 RNA was quantifiable in 60% (6 of 10) of the samples tested. In only one sample, the calculated value of the ultrasensitive assay was greater than 400 HIV-1 RNA copies/ml, and the variability of the assay would account for the difference observed (452 copies/ml for sample 4). No association of the viral load level obtained in the ultrasensitive assay can be made with either the OD of the samples or the OD variation above background.

Clinical specimens which were quantified as less than the detection limit of the Quantiplex HIV RNA 2.0 assay (bDNA) (Chiron Corporation, Emeryville, Calif.) (500 HIV-1 RNA copies/ml) were also evaluated in the ultrasensitive assay. The samples chosen for this study were also biased toward a likelihood of being >50 HIV-1 RNA copies/ml. For the Quantiplex assay, this was done by selecting samples which were not quantifiable but had relative luminescence (RL) units in the assay greater than the RL units for the standard D (STD D) of the test. The assumption was again that samples with RL readings greater than the STD D may actually contain HIV-1 RNA at levels that are not measured by the Quantiplex assay. Ten samples chosen by these criteria were evaluated in the ultrasensitive assay. A summary of the data is presented in Table 4. The ultrasensitive assay was able to measure HIV-1 RNA in

TABLE 2. Dilution series measured in the ultrasensitive Amplicor HIV-1 RNA Monitor test

| Sample and dilution | Expected | | Observed | | % obs/exp[a] |
|---|---|---|---|---|---|
| | $\text{Log}_{10}$ (copies/ml) | No. of copies/ml | $\text{Log}_{10}$ (copies/ml) | No. of copies/ml | |
| A | | | | | |
| 1:1 | 5.8 | 680,000 | 5.5 | 275,000 | 95 |
| 1:2 | 5.5 | 340,000 | 5.4 | 240,000 | 98 |
| 1:4 | 5.2 | 170,000 | 5.1 | 115,000 | 98 |
| 1:8 | 4.9 | 85,000 | 4.9 | 72,500 | 100 |
| B | | | | | |
| 1:1 | 5.8 | 592,000 | 5.5 | 310,000 | 95 |
| 1:2 | 5.5 | 296,000 | 5.3 | 200,000 | 96 |
| 1:4 | 5.2 | 148,000 | 5.0 | 104,500 | 96 |
| 1:8 | 4.9 | 74,000 | 4.7 | 49,000 | 96 |
| C | | | | | |
| 1:2 | 3.4 | 2,555 | 3.3 | 2,194 | 97 |
| 1:4 | 3.1 | 1,278 | 3.1 | 1,328 | 100 |
| 1:8 | 2.8 | 639 | 2.9 | 712 | 104 |
| 1:16 | 2.5 | 319 | 2.5 | 319 | 100 |
| 1:32 | 2.2 | 160 | 2.1 | 123 | 95 |
| 1:64 | 1.9 | 80 | 2.0 | 101 | 105 |
| 1:128 | 1.6 | 40 | 1.3 | 21 | 81 |

[a] % obs/exp, percent observed of total expected. Values were calculated with HIV-1 RNA $\log_{10}$(copies per milliliter).

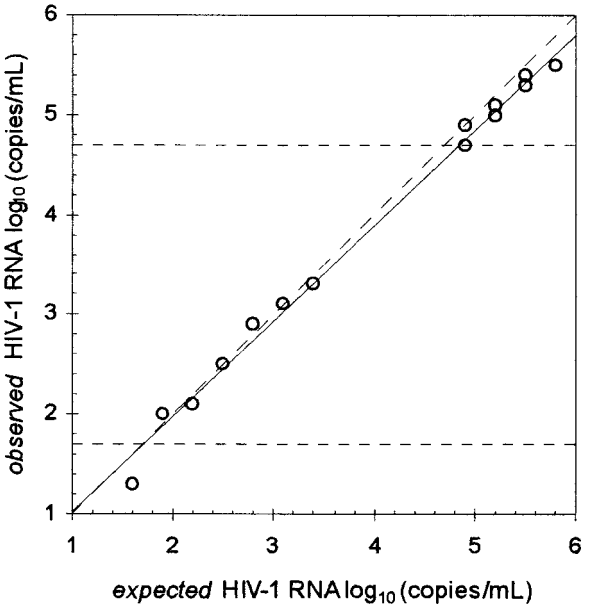


FIG. 1. Scatter plot of observed versus expected HIV-1 RNA $\log_{10}$(copies per milliliter) for the data presented in Table 2. The equation for the regression line (solid line) is $y = 0.958x + 0.054$, $r^2 = 0.994$, $r = 0.997$. The diagonal dashed line is the line of unity. The upper and lower horizontal dashed lines are 50,000 and 50 copies/ml, respectively.

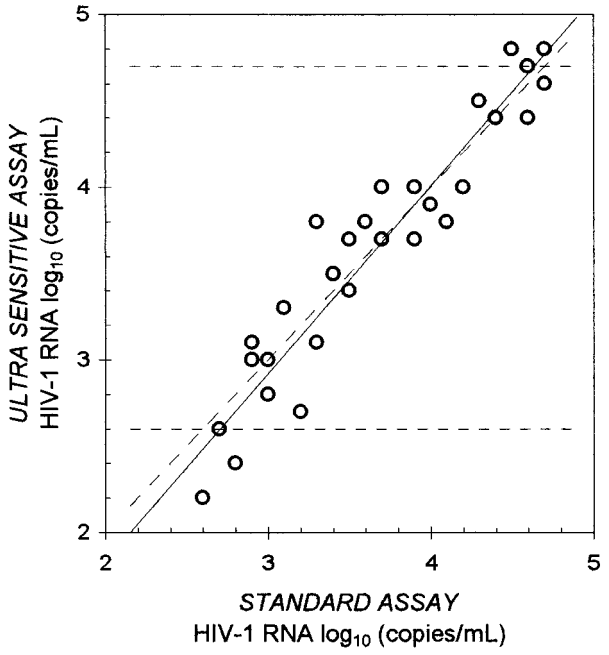


FIG. 2. Scatter plot of HIV-1 RNA $\log_{10}$(copies per milliliter) as determined by the ultrasensitive and standard Amplicor HIV-1 Monitor tests for 32 clinical specimens. The equation for the regression line (solid line) is $y = 1.088x - 0.343$, $r^2 = 0.904$, $r = 0.951$. The diagonal dashed line is the line of unity. The upper and lower horizontal dashed lines are 50,000 and 400 copies/ml, respectively.

100% (10 of 10) of the samples tested. In 7 of 10 samples, the calculated viral load was greater than 500 HIV-1 RNA copies/ml. Other studies have noted a similar disparity between Quantiplex and Amplicor results (4, 8). No conclusions can be drawn from these results regarding the accuracy or validity of either assay.

The widespread utilization of viral load testing for the evaluation and monitoring of HIV-1-infected patients has led to a rapid generational improvement in assay technology. A key modification has been the enhancement of assay sensitivity to levels which approach the limits imposed by the statistics of sampling, i.e., 20 to 50 HIV-1 RNA copies/ml. The early clinical experience with ultrasensitive HIV-1 testing supports the ability of these assays to discriminate treatment outcomes and provides an indication for routine testing (1).

TABLE 3. Ultrasensitive quantification of samples not detected[a] by the standard Amplicor HIV-1 Monitor test

| Sample | Ultrasensitive Amplicor HIV-1 Monitor test (HIV-1 RNA copies/ml) | Standard Amplicor HIV-1 Monitor test | |
|---|---|---|---|
| | | $OD_{450}$[b] | OD U above background[c] |
| 1 | <50 | 0.171 | 0.101 |
| 2 | <50 | 0.107 | 0.037 |
| 3 | <50 | 0.278 | 0.208 |
| 4 | <50 | 0.288 | 0.218 |
| 5 | 51 | 0.146 | 0.076 |
| 6 | 181 | 0.247 | 0.177 |
| 7 | 211 | 0.133 | 0.063 |
| 8 | 230 | 0.184 | 0.114 |
| 9 | 330 | 0.216 | 0.146 |
| 10 | 452 | 0.410 | 0.340 |

[a] The detection limit of the Amplicor HIV-1 Monitor test is 400 copies/ml.
[b] Sample OD at 450 nm for a dilution factor of 1 in the assay detection step.
[c] Calculated by using sample OD − 0.070.

TABLE 4. Ultrasensitive quantification of samples not detected[a] by Quantiplex HIV RNA 2.0 assay (bDNA)

| Sample | Ultrasensitive Amplicor HIV-1 Monitor test (HIV-1 RNA copies/ml) | Quantiplex HIV RNA 2.0 assay (bDNA) | |
|---|---|---|---|
| | | RL U[b] | RL U above STD D[c] |
| 1 | 190 | 0.862 | 0.084 |
| 2 | 350 | 0.750 | 0.241 |
| 3 | 470 | 0.553 | 0.149 |
| 4 | 1,200 | 0.858 | 0.003 |
| 5 | 1,400 | 0.950 | 0.167 |
| 6 | 1,500 | 0.648 | 0.178 |
| 7 | 1,600 | 0.709 | 0.174 |
| 8 | 1,900 | 0.919 | 0.331 |
| 9 | 2,200 | 0.868 | 0.238 |
| 10 | 2,300 | 0.742 | 0.111 |

[a] The detection limit of the Quantiplex HIV RNA 2.0 assay (bDNA) is 500 copies/ml.
[b] The mean RL of the sample in the assay.
[c] Calculated by using sample RL − STD D RL.

This study supports the reliability of the Amplicor HIV-1 Monitor test for the measurement of very low viral load levels. The ultrasensitive assay showed good within-run and between-run precision, and the dilution studies confirmed as reliable the stated reportable range of 50 to 50,000 HIV-1 RNA copies/ml.

An important issue for users of the Roche HIV-1 quantification system is the ability of the standard and ultrasensitive tests to give concordant results within their overlapping but nonidentical dynamic ranges. Although the two assays utilize essentially the same reagents for extraction, amplification, and detection, the ultrasensitive test depends on a highly efficient ultracentrifugation step in order to match results with the standard assay. This study finds an acceptable correlation between the ultrasensitive and the standard assays in the overlapping range between 400 and 50,000 HIV-1 RNA copies. Although small differences may exist in the absolute detection efficiencies of the two tests, the intrinsic sample-to-sample variation of either assay probably overshadows these differences. Careful attention to the details of sample preparation and proper centrifuge maintenance will contribute to good test concordance.

Although the current generation of commercial HIV-1 viral load tests achieves good precision over a broad dynamic range, they have not been cross-standardized for accuracy of RNA quantitation. Therefore, current recommendations for clinical testing discourage the comparison of results obtained in different assay systems. The development of ultrasensitive HIV-1 RNA assays further emphasizes the need for quantitative standardization. Examination of a subset of Quantiplex-negative samples with detectable but below-threshold signals (Table 4) allows the identification and comparison of clinical samples at the low end of HIV viremia. The data comparing Quantiplex and ultrasensitive Amplicor results, shown in Table 4, are in poor agreement and indicate that, at low levels of HIV viremia, different methodologies may produce very discrepant values. This supports the need for standardized calibration of viral load assays across the reportable range of the tests.

Efficient physician utilization of the Roche HIV-1 quantification assays depends on proper test selection. Since the great majority of patients who present for an initial HIV-1 viral load determination have levels above 400 HIV-1 RNA copies/ml, testing may begin with the standard assay. When the baseline

viral load is <50,000 HIV-1 RNA copies/ml or when that level is achieved in response to therapy, testing can continue with the ultrasensitive assay without the need to obtain a new baseline. During the first 8 months of ultrasensitive testing in our laboratory, approximately 15% of the results reported for the ultrasensitive assay exceeded 50,000 copies/ml. Careful attention to the levels and direction of viral load change is key to good patient management as well as cost-effective utilization of this test. The future development of ultrasensitive assays with extended dynamic ranges will simplify test utilization.

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