The 'Triple Study': viral dynamics and immune reconstitution in HIV-1 infection during potent antiretroviral therapy
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CHAPTER VIII

EVALUATION OF A SECOND GENERATION NASBA-BASED ASSAY FOR QUANTIFICATION OF HIV-1 RNA: COMPARISON TO PCR-BASED AND SIGNAL AMPLIFICATION BASED ASSAYS AND USE OF ULTRASENSITIVE PROTOCOL ADAPTATIONS

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Accurate assessment of HIV-RNA levels at low concentrations is clinically important. We evaluated a second-generation quantitative HIV-RNA assay (NucliSens HIV-1 QT), and three simple adaptations of the NucliSens standard protocol to lower the lower cutoff level. The assays were evaluated in constructed panels with known HIV-RNA concentrations and in clinical samples. Results were compared to those obtained with the first generation (NASBA HIV-1 QT) and to two other commercially available assays: the Amplicor HIV Monitor Test and the Quantiplex assay.

In a constructed panel, results obtained by NASBA QT were on average 0.13 log copies/mL (SD 0.15) higher than those of NucliSens. The NucliSens assay could quantify HIV RNA to a lower level than NASBA QT. Both assays correlated well to the known input (R NucliSens = 0.99; R NASBA QT = 0.996), but results were more variable at lower input levels. With the three different ultrasensitive NucliSens adaptations, HIV RNA could be quantified in at least 50% of the samples down to 100 (2.00 log(10)), 46 (1.66 log(10)), and 10 (1.00 log(10)) copies/mL, respectively. In patient samples, Amplicor results were on average 0.11 (SD 0.20) log copies/mL above, NucliSens 0.02 (SD 0.29) above and Quantiplex 0.13 (SD 0.19) below the mean of the three assay results per sample. The variation remained the same over the range of RNA levels with all three assays.

The NucliSens assay can quantify HIV-RNA at lower levels than the NASBA QT and is comparable to other commercially available assays. The lower cutoff of the NucliSens can be lowered down to 10 copies/mL.

INTRODUCTION

In persons with established HIV-1 infection, the level of viral RNA in plasma is considered a reflection of viral production and clearance. The plasma level of HIV-1 RNA is predictive for disease outcome from the first year after seroconversion. Reduction of HIV-1 RNA levels with antiretroviral therapy reduces the risk of developing AIDS. With the use of potent antiretroviral combination therapy, HIV-RNA levels decline to below the lower cutoff level of available assays within several weeks to months and these low levels can be sustained for months to years. Assays with increased sensitivity at low RNA levels, so called Ultrasensitive assays, are used in increasing frequency in clinical trials and routine patient care. The lowest RNA level that can be achieved in an ultrasensitive assay with antiretroviral therapy is indicative for the success of therapy.

Several assays for quantification of HIV-RNA levels are commercially available. Methods of HIV-RNA isolation, amplification and quantification differ between the assays, which may have implications for their clinical use. The Amplicor HIV Monitor assay is based on reverse transcriptase (RT) PCR, the NASBA HIV-1 QT is based on isothermal RNA amplification and the Quantiplex on branched DNA (bDNA) signal amplification.
generations of these assays have been extensively evaluated and compared and showed a high inter-assay correlation and reproducibility within clinically relevant limits.\textsuperscript{[19-22]}

To improve the quantification of low RNA levels, new generations of the assays have been developed and assay-protocols have been modified. In this paper we present results of the performance of a new version of the NASBA based assay, the NucliSens HIV-1 QT, in comparison with the NASBA HIV-1 QT, tested in a constructed panel, and in comparison with the Amplicor version 1.0 and Quantiplex version 2.0 in clinical samples. All three assays have a lower cutoff level in the same order of magnitude. In addition, we evaluated the performance of three ultrasensitive protocol adaptations of the NucliSens in constructed panels and clinical samples.

MATERIALS AND METHODS

Samples

Different sample sets were used for different evaluations. The performance of the different NASBA-based assay versions and ultrasensitive protocol adaptations were evaluated in constructed panels of serial dilutions with known HIV-RNA concentrations. The evaluation of clinical performance and comparisons with other commercially available assays were done on patient samples from two different antiretroviral therapy trials.

Constructed panels

For the comparison of NucliSens with NASBA QT a panel was constructed using an HIV-1 subtype B RNA standard, with electron microscopy counted viral particle numbers, from the Viral Quality Assurance Lab, Rush-Presbyterian-St.Luke's Medical Center, Chicago, IL, USA.\textsuperscript{[23]} Samples were prepared in bulk by dilution in human EDTA-plasma pre-mixed with lysis buffer, resulting in a sample set containing 6.91, 6.21, 5.51, 4.81, 4.46, 4.11, 3.76, 3.41, 3.06, 2.71, 2.37, 2.02 and 0 log\textsuperscript{i} copies/mL, respectively. For all dilutions, NASBA QT and NucliSens were each performed in duplicate in five different laboratories experienced with the assay procedures: the Department of Human Retrovirology, AMC, Amsterdam; the Department of Viral Diagnostics, CLB Sanquin Blood Supply Foundation, Amsterdam, The Netherlands; the Laboratory of Virology, Retrovirus Section, Division of Antiviral Therapy, the Instituto Superiore di Sanita, Rome, Italy; the Medizinisch Immunologisch Laboratorien, München, Germany; the Department of Virology, Glaxo Wellcome Inc, Research Triangle Park, NC, USA.

From the same viral stock a second panel was made containing 3.41, 2.71, 2.02, 1.66, 1.32, 1.00 and 0 log\textsuperscript{i} copies/mL, respectively. This was used to evaluate the NucliSens assay using a ten-fold increase in sample input (2 mL plasma). These samples were also tested in duplicate in the same five labs.

A third panel was made to evaluate the Ultra-NucliSens protocols, containing 3.70, 2.90, 2.60, 2.30 and 2.00 log\textsuperscript{i} copies/mL, respectively, to
test the Ultra-NucliSens assay using 0.2 mL input and 1.90, 1.60, 1.30 and 1.00 copies/mL, respectively, to test the Ultra-NucliSens assay using 2 mL input. These samples were tested at Organon-Teknika BV, Boxtel, The Netherlands.

**Antiretroviral therapy trial I samples**

NucliSens was compared with Amplicor and Quantiplex and the use of the Ultra-NucliSens was evaluated using frozen EDTA-plasma samples from the 33 participants in a triple therapy study, using a protease inhibitor with two nucleoside analogue RT inhibitors. After screening-assessment, blood was sampled three times pre-treatment, eight times during the first eight weeks of treatment and every four weeks for the remaining first year of treatment.

All samples of all participants, while included in the study, were tested using Amplicor, and at a later time point NucliSens was performed on stored samples. Samples from the first four weeks of treatment were also tested with Quantiplex. In addition, samples were tested with Ultra-NucliSens if regular NucliSens (or if NucliSens not performed, Amplicor) result was below the lower cutoff.

**Antiretroviral therapy trial II samples**

Four NASBA-based assays: The NASBA QT, the NucliSens, and two ultrasensitive protocol adaptations with increasing sensitivity, the Ultra-NucliSens and a 2mL-Ultra-NucliSens, were tested in study-participants using an antiretroviral quintuple combination. NucliSens was used when NASBA QT results were below the lower cutoff or not available, Ultra-NucliSens was used when NucliSens results were below the lower cutoff and 2mL-Ultra-NucliSens when Ultra-NucliSens results were below the lower cutoff.

The Quantiplex bDNA assay was performed at Chiron Diagnostics Laboratories, Amsterdam, The Netherlands. All other tests for the two antiretroviral therapy trials were performed at the Department of Human Retrovirology, AMC, Amsterdam, The Netherlands.

**Quantitative HIV-RNA assays**

*NASBA-based RNA amplification*

Tests used were NASBA HIV-1 RNA QT (NASBA QT) and NucliSens HIV-1 QT (NucliSens) (Organon Teknika BV, Boxtel, The Netherlands), based on isothermal amplification of RNA and ultrasensitive protocol adaptations of the NucliSens assay (Table 1).

For the standard assay versions, viral RNA was isolated from 100 µL of sample for the NASBA QT or from 200 µL for the NucliSens, with GuSCN containing lysis buffer and a silica suspension to bind nucleic acids. Three synthetic calibrator RNA molecules, Qa, Qb and Qc, with different probe-binding regions, were each added in different concentrations. Subsequently, RNA was eluted using 50 µL of Tris-EDTA based elution buffer. For both NASBA QT and NucliSens, 5 µL of this nucleic acid solution was used for amplification. WT sample and calibrator amplification products were detected.
by electrochemiluminescence (ECL) with different ruthenium-labeled probes and streptavidin coated paramagnetic beads.

For NucliSens, lower concentrations of calibrators were used than for the NASBA QT to reach a lower linear quantification range. Furthermore, the software for computing RNA copies from the ECL signals from the different calibrators and wild-type products was slightly modified.

The NASBA QT and NucliSens allow for two possibilities to increase the sensitivity: the input of plasma or serum before RNA isolation can be increased up to ten-fold to 1 and 2 mL, respectively, and the amount of isolated RNA to be amplified can be increased up to a ten-fold. With the higher amount of patient sample, the amount of lysis buffer is increased proportionally. After addition of the silica, the volume of the RNA-containing suspension can be reduced back to that of the regular procedure (1 mL). For the second protocol adaptation, named the Ultra-NucliSens, RNA recovery during isolation is increased by a second elution of RNA from the silica and concentration of the eluted RNA by ethanol precipitation using Pellet Paint™ (Novagen, Madison, WI, USA) to visualize the RNA-pellet. The RNA is resuspended in 5 µL elution buffer. Both methods can be combined, thereby theoretically increasing the sensitivity a hundred-fold.

For the use in clinical samples, the NASBA QT lower cutoff level was set at 1000 copies/mL (3.00 log₁₀) and the NucliSens at 400 copies/mL (2.60 log₁₀). Lower cutoff levels of the Ultra-NucliSens and the 2mL-Ultra-NucliSens were derived from results of panel 3 in this study: the input-level that gave at least 50% positive results was regarded as lower cutoff level.

<table>
<thead>
<tr>
<th>Assay name</th>
<th>NASBA QT</th>
<th>NucliSens</th>
<th>2mL-NucliSens</th>
<th>Ultra-NucliSens</th>
<th>2mL-Ultra-NucliSens</th>
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<tr>
<td>Sample input</td>
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<td>200 µL</td>
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<td>Given input, Given input</td>
<td>Panel 3, Trial I &amp; II</td>
<td>Given input, Given input</td>
<td>Given input, Given input</td>
</tr>
<tr>
<td>Compared to</td>
<td>NASBA QT, Amplicor, Quantiplex, (2mL-Ultra-NucliSens)</td>
<td>NucliSens, Ultra-NucliSens</td>
<td></td>
<td>NucliSens, Ultra-NucliSens</td>
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</tr>
</tbody>
</table>

Table 1. A summary of the differences in the assay protocols of NASBA QT and NucliSens and comparisons made in this study. For the NucliSens assay lower concentrations of standard RNA molecules (Qs) were used than for the NASBA QT and the software for computing RNA copies from the ECL signals from the different Qs and WT products was slightly modified.
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RT-PCR

Test used was the Amplicor HIV Monitor Test version 1.0 (Roche Molecular Systems, Branchburg, NJ, USA). This test is based on PCR amplification of the sample of interest together with a quantification standard (QS) of known concentration amplified with biotin-labeled primers. The lower cutoff of the assay varies with the optical density of the amplified QS, after addition of avidin-horseradish peroxidase conjugate. According to the manufacturer the lower limit of the linear response of the test is 400 HIV-RNA copies/mL (2.60 log₁₀). We used the variable calculated lower cutoff that was usually below 400 copies/mL.

bDNA

Test used was the Quantiplex bDNA signal amplification assay (version 2.0) (Chiron Corporation, Emeryville, CA, USA), with a lower cutoff set at 500 copies/mL (2.70 log₁₀).⁵⁷

Statistical analysis

All results were log₁₀ transformed before further analysis. Results below the lower cutoff were assigned the lower cutoff level as result. For statistical analysis SPSS version 7.5 software (SPSS Inc., Chicago, IL, USA) was used.

For the evaluation of NucliSens and NASBA QT in the constructed panel, results and differences between the assays with each other or with the given input RNA level were correlated to the given input level. Intraclass correlation coefficients of reliability (ICC) and corresponding lower boundary of the 95% confidence interval (l.b.95%CI) were calculated for the duplicate measurements of the constructed panel with NucliSens and NASBA QT. The ICC of a series of duplicate measurements represents the ratio between the between-sample mean square (BMS) minus within-sample mean square (WMS) and the BMS plus WMS. An ICC value of 1 represents perfect reproducibility, whereas 0 represents complete non-reproducibility.²⁶

RESULTS

Performance in constructed panels

NucliSens and NASBA QT performance in panel 1

NucliSens and NASBA QT were evaluated in reconstructed panel 1, with input levels ranging from 6.91 to 2.02 log₁₀ copies/mL and a negative control. All samples were tested in duplicate by five different laboratories.

The higher sample input volume and lower concentrations of the calibrator molecules for NucliSens resulted in an increase in sensitivity compared to NASBA QT: for input levels ≥ 2.71 log₁₀ (518) copies/mL all but one NucliSens measurements were above the lower cutoff, while for NASBA QT at ≥ 3.76 log₁₀ (5796) copies/mL input all measurements were above the lower cutoff and at 3.41 log₁₀ (2592) copies/mL 8 of 10 were above the lower cutoff. Further analyses of the results were done on input levels that gave at least 80% of measurements above the lower cutoff for each assay.

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Both NucliSens and NASBA QT results correlated well with the given input level and with each other, although the variation of the NucliSens results, and to a somewhat lesser extent those of the NASBA QT, increased at the lower levels of the given standard input (Fig. 1A). For the NucliSens results, the Pearson’s correlation coefficient ($R_p$) with the input level was 0.99 ($p < 0.001$), with a linear regression slope of 0.99 and an intercept of 0.02. For the NASBA QT results, the $R_p$ with the input was 0.996 ($p < 0.001$), with a slope of 0.95 and an intercept of 0.38. For NucliSens compared with NASBA QT, the $R_p$ was 0.99 ($p < 0.001$), with a linear regression function of NucliSens = 1.02$x$ (NASBA QT) - 0.23.

The mean difference of the NucliSens results with the given input was +0.022 (SD 0.17, range -0.38 to 0.44) log$_{10}$ copies/mL for 99 available results. For the 78 NASBA QT measurements the difference was -0.12 (SD 0.12, range -0.37 to 0.10) log$_{10}$ copies/mL and this difference was significantly larger at lower input levels ($R_p$ of this difference with the actual input level was 0.53, $p < 0.001$). NASBA QT results were overall 0.13 log$_{10}$ copies/mL higher than the NucliSens results (SD 0.15, range -0.18 to 0.52). This difference was not significantly correlated to the input levels.

The mean difference between the duplicate runs was 0.02 log$_{10}$ copies/mL (SD 0.11) for NASBA QT and 0.06 (SD 0.20) for NucliSens. For neither assay the difference was significantly correlated to the input level. The ICC, as indication of reproducibility of the duplicate runs was 0.987 for NucliSens (l.b.95%CI: 0.978) and 0.995 for NASBA QT (l.b.95%CI: 0.991), for all input levels and the 5 labs combined. The ICC for the five labs was 0.971 for NucliSens (l.b.95%CI: 0.954) and 0.987 for NASBA QT (l.b.95%CI: 0.972).

2mL-NucliSens performance in panel 2

Increasing the input volume in the regular NucliSens assay a tenfold to 2mL resulted in quantification of all samples of the 2nd construction panel with an HIV-RNA concentration of at least 2.02 log$_{10}$ (104) copies/mL, and six out of ten with 1.66 log$_{10}$ (46) copies/mL (Fig. 1B).

Mean values were close to the known input level: 0.07 and 0.09 log$_{10}$ copies/mL below the input levels of 2.71 and 2.02 log$_{10}$ copies/mL, respectively. At the input of 1.66 log$_{10}$ copies/mL the deviation of the measured results from the input increased to +0.19 log$_{10}$ copies/mL. At the lower input levels variation increased, with a SD of the measured results of 0.19 log$_{10}$ copies/mL at an input of 2.71 log$_{10}$ copies/mL and 0.39 log$_{10}$ copies/mL at an input of 1.66 log$_{10}$ copies/mL.

Ultra-NucliSens and 2mL-Ultra-NucliSens performance in panel 3

The performance of the Ultra-NucliSens and the 2mL-Ultra-NucliSens assays, in which the isolated RNA was concentrated by precipitation before amplification, were evaluated in panels with low copy numbers to determine the lower cutoff levels. With the Ultra-NucliSens, at 2.00 log$_{10}$ (100) copies/mL 15 out of 29 (52%) and at 2.30 log$_{10}$ (200) copies/mL 15 out of 24 (62%) of the samples could be quantified. At levels down to 2.30 log$_{10}$ copies/mL, the mean difference of the Ultra-NucliSens results with each input
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Fig. 1. Measured results, in log_{10} copies/mL, versus known input of the reconstruction panels. Shown are mean values with standard deviations, from input levels that gave at least 50% results above the lower cutoff for each assay. A. NASBA QT and NucliSens. B. NucliSens results of input levels below 4.0 log_{10} copies/mL, and the three different ultrasensitive protocol variations.
level was between $+0.17$ and $+0.21 \log_{10}$ copies/mL; at $2.00 \log_{10}$ copies/mL the difference was $+0.44 \log_{10}$ copies/mL. At the lower input levels variation was considerable, with a SD of the measured results between 0.33 and 0.39 $\log_{10}$ copies/mL at input levels between 2.00 and 2.90 $\log_{10}$ copies/mL (Fig. 1B).

With the 2ml-Ultra-NucliSens, at the lowest input level tested, $1.00 \log_{10}$ (10) copies/mL, 10 out of 15 (67%) of the samples could be quantified and at $1.30 \log_{10}$ (30) copies/mL, 7 out of 8 (87%). At an input level of $1.00 \log_{10}$ copies/mL, the mean difference of the 2ml-Ultra-NucliSens results with the input level was $+0.53 \log_{10}$ copies/mL; at the higher input levels the mean difference was between $+0.10$ and $+0.15 \log_{10}$ copies/mL. The SD of the measured results was 0.40 and 0.44 $\log_{10}$ copies/mL at input levels of 1.00 and $1.30 \log_{10}$ copies/mL, respectively, and 0.22 and 0.26 $\log_{10}$ copies/mL at input levels of 1.60 and $1.90 \log_{10}$ copies/mL, respectively (Fig. 1B).

The lower cutoff level, defined as the input level that gave at least 50% positive results in the constructed panels, was set at $2.00 \log_{10}$ (100) and $1.00 \log_{10}$ (10) copies/mL for the Ultra-NucliSens and the 2ml-Ultra-NucliSens, respectively.

**Performance in clinical samples**

**NucliSens, Amplicor and Quantiplex in trial 1**

For the clinical evaluation of NucliSens, Amplicor and Quantiplex, results of all three assays were available in 147 samples from 30 of the participants in antiretroviral therapy trial 1, taken during the first four weeks of treatment. Of these 147 samples, 122 were above the lower cutoff in all three assays and were used for further analysis.

All three assays correlated with each other: for NucliSens with Quantiplex, $R_p$ was 0.87 ($p < 0.001$), for NucliSens with Amplicor, $R_p$ was 0.86 ($p < 0.001$) and for Amplicor with Quantiplex, $R_p$ was 0.95 ($p < 0.001$). NucliSens results were on average below Amplicor and above Quantiplex results.

To obtain reference values to compare the different assays, for each sample the mean of the results of the three assays, and for each measurement the difference to this mean were calculated (Fig. 2). Amplicor differed on average $+0.11$ (SD 0.20, range -0.73 to 0.37) $\log_{10}$ copies/mL, NucliSens $+0.016$ (SD 0.29, range -0.71 to 1.08) and Quantiplex -0.13 (SD 0.19, range -0.54 to 0.73) from the mean of the three (Fig. 2). These differences did not correlate to the levels of the mean HIV-RNA concentration per sample.

**NucliSens and Amplicor concordance of cutoff in trial 1**

Samples from participants in the same therapy trial with a longer follow-up period were used for analysis of NucliSens and Amplicor performance at RNA levels close to or below the lower cutoff of these assays. A total of 512 samples from 32 of the subjects were tested with both assays. The variable Amplicor lower cutoff had a mean value of $2.30 \log_{10}$ copies/mL (SD 0.19) for the 336 Amplicor measurements with results below the lower cutoff.

In 449 of the 512 samples (88%) both assays were in concordance with
regard to being both above or both below the lower cutoff. Of the 63 discordant samples, 14 were not truly discordant: in two samples, Amplicor results were below its lower cutoff level, while this lower cutoff was above the value of the NucliSens lower cutoff (2.60 log_{10} copies/mL) and twelve samples had quantifiable Amplicor results that were below the value of the NucliSens lower cutoff level. The concordance thereby increased to 463 samples (90%).

In 258 samples, RNA levels were above the lower cutoff in both assays. For these samples the correlation (R_p) between the two assays was 0.87 (p < 0.001). NucliSens gave on average slightly lower results than Amplicor (mean difference -0.11, SD 0.48 log_{10} copies/mL), however, the difference occasionally showed considerable variation, with a range of -1.95 to 1.14 log_{10} copies/mL. The difference between Amplicor and NucliSens results did not correlate with the levels of the mean HIV-RNA of the two assays concentration per sample (R_p = -0.13, Fig. 3).

![Graph](image_url)

**Fig. 2. Amplicor, NucliSens and Quantiplex results** The difference of each measurement with one of the three assays plotted against the mean of the results of the three assays per sample. Regression lines were fit per assay. Shown are results of 122 samples from 25 subjects with HIV-RNA levels above the lower cutoff in all three assays.
Ultra-NucliSens following unquantifiable NucliSens results in trial I

The Ultra-NucliSens procedure was performed if NucliSens results were below its lower cutoff, in participants of trial I. Samples were analyzed when measurements were performed with at least two assays (NucliSens, Amplicor or Ultra-NucliSens) from subjects on full study treatment for 24 to 52 weeks. From 26 subjects 431 samples were available, in 255 of which Ultra-NucliSens measurements were performed (Fig. 4).

Fig. 3. Amplicor versus NucliSens. The difference between the result of each of the two assays plotted against the mean result of the two assays for each sample. Shown are results from 258 samples from 32 subjects with HIV-RNA levels above the lower cutoff in both assays.

In 204 of the 252 samples with Amplicor and/or NucliSens results below the lower cutoff, Ultra-NucliSens was below its lower cutoff as well, while in 48 Ultra-NucliSens was above the lower cutoff. The median time (25- and 75-percentiles) to HIV-RNA levels persistently below the lower cutoff in each assay was 6 (4 - 8), 12 (8 - 12) and 12 (8 - 15) weeks for the NucliSens, the Amplicor and the Ultra-NucliSens assay, respectively.

NucliSens ultrasensitive protocols in trial II

Four different NASBA-based assay variations, NASBA QT, NucliSens, Ultra-NucliSens and 2mL-Ultra-NucliSens were used in Antiretroviral therapy trial II, in a consecutive manner: starting with NASBA QT, when results were below the lower cutoff, the next more sensitive assay was used. The time to reach RNA levels below the lower cutoff increased with the decreasing lower cutoff (Fig. 5). At week 8, eight out of ten (80%) had RNA levels below the Ultra-
NucliSens lower cutoff and two out of nine (22%) below the 2mL-Ultra-NucliSens cutoff, while at week 16 this was seven out of eight (89%) with the Ultra-NucliSens and seven out of ten (70%) with the 2mL-Ultra-NucliSens assay.

\[
\begin{array}{ccccccc}
\text{Weeks on therapy} & 0 & 4 & 8 & 12 & 16 & 20 & 24 & 28 & 32 & 36 & 40 & 44 & 48 & 52 \\
\text{HIV-RNA (log_10 copies/ml)} & 6.0 & 5.5 & 5.0 & 4.5 & 4.0 & 3.5 & 3.0 & 2.5 & 2.0 & \text{Lower cutoff levels} \\
\text{NucliSens} & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash \\
\text{Amplicor} & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash \\
\text{Ultra-NucliSens} & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash & \cdashdash \\
\end{array}
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**Fig. 4. Amplicor, NucliSens and Ultra-NucliSens in clinical trial I.** Median HIV-RNA levels with 25- and 75-percentiles, per week on treatment, with the percentage of results below the lower cutoff for each assay. Ultra-NucliSens was performed if NucliSens results were below the lower cutoff. Shown are results from 26 subjects on full study treatment for 24 to 52 weeks, when measurements were performed with at least two assays.

**DISCUSSION**

We evaluated the NucliSens HIV-1 QT assay, its predecessor, NASBA HIV-1 QT, and several ultrasensitive NucliSens protocol adaptations for their performance in quantification of HIV-1 RNA in constructed panels with known HIV RNA levels. Furthermore, NucliSens and the ultrasensitive protocols were evaluated in clinical samples and NucliSens was compared with two other commercially available assays, Amplicor and Quantiplex, in a clinical setting.

Overall NucliSens and NASBA QT results from the construction panel were comparable and correlated well to the input HIV-RNA concentration. Results from both assays showed high overall reproducibility for the duplicate runs as well as for the replicate tests between the different labs. NASBA QT and to a larger extent NucliSens varied more at lower input levels, but mean NucliSens levels were closer to the actual input levels. NASBA QT resulted in slightly higher HIV-RNA levels than NucliSens (mean difference +0.13 log_{10}

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copies/mL). In recent studies, a comparable performance of NucliSens was reported.\textsuperscript{27,28}

The NucliSens assay allows for lowering of the lower cutoff by different protocol adaptations: using a larger input volume of 2 mL, or concentrating the RNA after isolation, and both methods can be combined. In the constructed panels, the 2mL-NucliSens assay could quantify at least 50% of samples at input levels down to approximately 46 (1.66 $\log_{10}$) copies/mL. For the Ultra-NucliSens this was 100 (2.00 $\log_{10}$) copies/mL and for the 2mL-Ultra-NucliSens 10 (1.00 $\log_{10}$) copies/mL. At these levels, however, the measured results deviated considerably from the given input and a more linear relationship goes down only to 104 (2.02 $\log_{10}$), 200 (2.30 $\log_{10}$) and 20 (1.30 $\log_{10}$) copies/mL for the 2mL-NucliSens, Ultra-NucliSens, and 2mL-Ultra-NucliSens assay, respectively. A slightly different protocol adaptation of 2mL-Ultra-NucliSens was able to quantify HIV RNA at concentrations down to approximately 10 to 20 copies/mL in a constructed panel.\textsuperscript{29}

![Graph](image)

**Fig. 5.** NASBA QT, NucliSens and Ultra-NucliSens and 2 mL-Ultra-NucliSens in clinical trial I. Results in twelve participants from a pentuple combination study, trial II. Median results for visits with at least three participants tested per assay, with number tested with each assay.

In the analysis of three commercially available assays in clinical samples, NucliSens results were on average in between Amplicor and Quantiplex results. In an expanded set of samples tested with these two assays, the
difference between NucliSens and Amplicor was comparable. Previous comparisons between the different assays reported higher HIV-RNA results when measuring with NASBA QT than with Amplicor and found the lowest results for Quantiplex.\textsuperscript{19,21,30} The differences of the assays with the known input levels and the differences between the assays indicate that a variation up to approximately 0.5 \( \log_{10} \) copies/mL between two assessments, both with the same or with different assays, commonly occurs and should therefore not be considered clinically significant. This is in accordance to the variation reported in other studies\textsuperscript{21,31}

The relatively small difference between the assays allows comparison between groups of samples, for instance from different antiretroviral treatment studies, tested with different assays. However, occasionally the individual difference can be considerable, therefore switching to different assays within studies or within individual follow-up should be avoided whenever possible. Furthermore, the analysis in the constructed panels showed that the assay results varied significantly at lower RNA levels, that are critical for decisions related to starting or switching antiretroviral therapy.\textsuperscript{13} If the result is too close to a threshold for clinical decisions, a second measurement is warranted.

Several factors can influence HIV-RNA results, causing random variation and/or structural differences between the assays. Some of these factors will be of more importance at low RNA copy numbers, like sampling errors and chance events in primer annealing and other biochemical reactions involved. By repetitive testing of the same samples, test and sampling variation can be distinguished from the biological variation over time. Usage of serum or plasma, and type of anticoagulant used, can be another cause of differences.\textsuperscript{22,31,32} The use of different isolation and amplification methods, primer binding sites and quantification standards can result in consistent differences between the assays.

In conclusion, with the NucliSens assay HIV-RNA can be quantified at lower levels than with the previous generation, the NASBA QT. The NucliSens is comparable to two other widely used commercially available assays, the Amplicor and Quantiplex. Up to three-fold variation (0.5 \( \log_{10} \)) between laboratories, assays and repeated measurements is possible and should not be considered clinically significant. With ultrasensitive protocol adaptations the quantification limit of the NucliSens can be lowered to 100 copies/mL and further down to 10 copies/mL.

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