

Evaluation of the NucliSens EasyQ assay in HIV-1-infected individuals in South Africa

W. Stevens*, T. Wiggill, P. Horsfield, L. Coetzee, L.E. Scott

Department of Molecular Medicine and Haematology, School of Pathology, Faculty of Health Sciences, University of Witwatersrand and the National Health Laboratory Service, York Rd., Parktown 2193, South Africa

Received 19 August 2004; received in revised form 9 November 2004; accepted 10 November 2004
Available online 15 December 2004

Abstract

We compared the performance of the NucliSens EasyQ assay (bioMérieux) combined with the manual NucliSens miniMag extraction methodology to the Roche Cobas Ampliprep/Standard Amplicor Monitor methodology (Roche Diagnostics) for HIV-1 RNA quantitation in HIV-1-infected individuals in South Africa. Plasma samples (284) from HIV sero-positive patients at different stages of infection were analyzed. The distribution of results was typical of the clinical samples received at the laboratory where 20% have viral load results <400 copies/ml (2.6 log) and 18% have viral load results >750 000 copies/ml (5.8 log) using the Roche Amplicor Monitor standard assay. All statistical analyses were performed using log₁₀-transformed values for all the variables in the analyses, i.e. log₁₀EasyQIU/ml, and log₁₀RNA (log₁₀ copies/ml, Amplicor). Roche values were converted from RNA copies per ml to IU/ml by multiplying the Roche value by 0.51. HIV RNA levels quantitated by the NucliSens EasyQ assay correlated significantly with those of the Roche Cobas Amplicor Monitor assay ($r=0.874$, $p<0.0001$). Reproducibility of the NucliSens EasyQ assay in the log₆ IU range yielded CV variance of 1.3–2.84% for two well-trained technologists. In addition, a retrospective evaluation of the performance of the NucliSens EasyQ assay in 102 runs (2448) samples was conducted in the laboratory over a 4-month interval. Factors considered during this evaluation included time taken to perform the assay, volume requirements, number of required repeats, potential for contamination
© 2004 Elsevier B.V. All rights reserved.

Keywords: HIV viral load laboratory measurement; NucliSens EasyQ; Roche Amplicor; Method comparison; Resource limited setting

1. Introduction

Plasma viral load monitoring is considered an integral part of the standard of care for HIV-infected patients in first world settings. Much debate has ensued locally and internationally around the value of these assays for treatment initiation and monitoring in resource poor settings and, in fact, these assays are classified as optional under the draft WHO guidelines for implementation of anti-retroviral therapy ([http://www.iapac.org/text/pdf/WHO, 2002](http://www.iapac.org/text/pdf/WHO_2002)). In South Africa, numerous different viral load testing strategies have been extensively evaluated but multiplied by

the number of patients requiring access to therapy this results in a staggering total cost that most industrialized countries would be hard pressed to afford. Similar to the pressure placed on pharmaceutical organizations to reduce costs of anti-retroviral agents, pressure has been placed on diagnostic companies to reduce the costs of nucleic acid monitoring strategies in resource-constrained environments.

Commercial testing platforms available for viral load monitoring include: reverse-transcriptase PCR (RT-PCR), nucleic acid sequence-based amplification (NASBA) and the branched-DNA (bDNA) and ligase chain reaction (LCR) (Dyer et al., 1999; Ginocchio et al., 1999; Murphy et al., 2000). A more affordable option that has been recently introduced in South Africa, but has not yet undergone extensive

* Corresponding author. Tel.: +27 11 489 8505; fax: +27 11 484 5812.
E-mail address: wendy.stevens@nhls.ac.za (W. Stevens).

clinical validation, is the NucliSens EasyQ HIV-1 assay version 1.1 from bioMerieux (Boxtel, The Netherlands). This assay is a real-time NASBA using molecular beacon-based detection technology (Weusten et al., 2002). The assay consists of a two-step process, namely, nucleic acid amplification combined with a homogenous detection step. The assay differs considerably from previous versions of the NucliSens QT assay in that: (1) the PCR is real-time versus endpoint detection, (2) a single fixed amount of RNA calibrator is added as an internal control compared to previous addition of three different quantitation standards/calibrators, (3) detection is fluorescence-based using molecular beacons versus electrochemiluminescence and (4) a new semi-automated preparation step called NucliSens miniMag has been developed to enhance throughput. Currently, little published data on the performance of this assay in (1) HIV-1 Subtype C specimens is available (Segondy et al., 2002) and (2) the combination of this assay with the semi-automated NucliSens miniMag extraction procedure (combination of Boom chemistry and magnetic silica particles) has also not been extensively validated.

The evaluation was conducted in two phases: (1) direct correlation of assay performance with the Roche Amplicor version 1.5 standard assays (Roche diagnostic Systems, Branchburg, NJ, USA) and (2) qualitative assessment of the assay performance in the laboratory over a period of several weeks.

2. Materials and methods

2.1. Sample collection

A total of 284 samples (from individual patients) were randomly selected from HIV-1 positive individuals followed up routinely at the Johannesburg Academic hospital in the Gauteng province in South Africa. No previous viral load results were recorded from these patients, and thus, longitudinal follow-up could not be included in this study. The HIV-1 subtype for this population was not determined but is accepted as HIV-1 subtype C from this southern African location (Papathanasopoulos et al., 2003; Scriba et al., 2001; van Harmelen et al., 2001). Studies were conducted in full conformance with the local ethics committee approval. Clinical specimens were fresh and/or frozen plasma collected in vacutainer EDTA tubes. HIV viral RNA was extracted from each specimen using two different extraction procedures: (1) the Ampliprep/Amplicor HIV-1 Monitor assay (version 1.5) (Roche diagnostic Systems, Branchburg, NJ) as per manufacturer's instructions and (2) for the NucliSens EasyQ assay (bioMerieux, Boxtel, The Netherlands), a 1 ml input volume of plasma was used and added to 2 ml of lysis buffer prior to analysis. All staff were trained by the respective manufacturer's prior to start of assay evaluation.

2.2. Quantification of RNA

2.2.1. Roche Amplicor version 1.5 (RT-PCR)

The assay validation was conducted using the standard version of the assay, which has lower and upper detection limits of 400 and 750 000 RNA copies/ml, respectively. The input volume of this assay is 350 μ l and uses a 70 μ l extraction volume. Results below the detection limit of the standard Roche assay were not evaluated further using the ultra-sensitive version of the kit.

2.2.2. NucliSens EasyQ assay (NASBA)

This assay has a reported dynamic range of 25–3 million IU/ml of plasma. The only changes made were the addition of negative and positive control samples to the run, which are not routinely supplied by the manufacturer. Extractions were performed using an input volume of 1 ml with an internal calibrator added to each sample prior to the extraction. Purified HIV-1 RNA and internal calibrator RNA were co-amplified and amplicon formation was measured in real-time on the EasyQ analyzer. In cases, where undetectable levels of viral load were obtained in both assays, they were considered concordant.

2.3. Reproducibility

The reproducibility of the EasyQ assay was assessed using two pooled plasma samples (one high and one low viral load values). Two different technologists performed each pair of samples five times and the standard deviation and %CV were calculated.

2.4. Input sample volume

The EasyQ assay has been validated using an input volume of 1 ml. We conducted a series of dilutions on two specimens (log 6 IU/ml, log 4 IU/ml) to ascertain the contribution of input volume to the reproducibility of viral load quantitation results.

2.5. Retrospective evaluation of data

In addition, a retrospective analysis was conducted of 102 runs performed in the laboratory over the period April to July 2004. A total of 102 runs of 24 samples each were available for analysis. The following information was recorded: (1) total volume of sample submitted by clinicians, (2) number of invalid results reported on the analyzer, (3) performance of controls and (4) time taken to conduct the assay.

2.6. Statistical analysis

All statistical analyses were performed using log₁₀-transformed values for all the variables in the analyses, i.e. log₁₀Easy IU/ml, and log₁₀RNA (log₁₀ copies/ml, Amplicor). Roche values were converted from RNA copies/ml

to IU/ml by multiplying the Roche value by 0.51 according to the EasyQ supplier's instruction booklet. A frequency distribution was used to describe the data set as well as the mean, median and minimum and maximum range for both assays. All additional statistics were performed using the Roche transformed data. Correlation was used to determine the linear relationship between the assay values, and linear regression quantitates this relationship. The coefficient of determination (R^2) as the square of the correlation between the observed and predicted values was also calculated. Method comparison to determine agreement between the two assays was analyzed by Bland and Altman (1986) and percentage similarity (Scott et al., 2003) models. All statistical tests were two-sided and all statistical analyses were performed using the SAS V8.2 Enterprise Guide V2 software and GraphPad Prism software version 4.02.

3. Results

3.1. Summary statistics

A total of 284 results were available for direct comparison between the two assays. Fig. 1 describes this data set with 38% of the samples in the upper and lower limits of the Roche AmpliCor viral load results. The mean log Roche AmpliCor viral load (converted to log IU/ml) for this data set is 4.37 IU/ml (2.31–5.71 IU/ml) and the mean log EasyQ viral load result is 4.31 IU/ml (1.4–6.71 IU/ml). The EasyQ has a broader range (25–5000 000 IU/ml) than the Roche AmpliCor (204–382 500 IU/ml: 400–750 000 RNA copies/ml). This distribution is typical of the clinical samples received at the laboratory where 20% have viral load results <400 copies/ml (2.6 log) and 18% have viral load results >750 000 copies/ml (5.8 log). The sensitivity of the Roche assay may be improved by using the ultrasensitive version of the kit, which has a dynamic range of 50–100 000 RNA copies/ml.

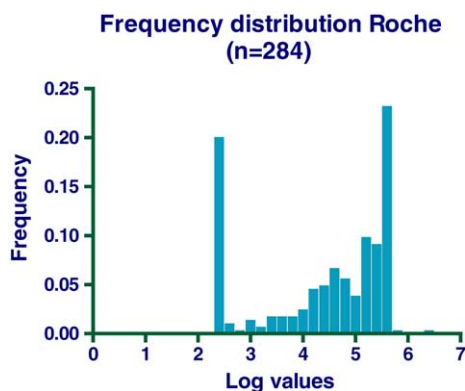


Fig. 1. A frequency distribution of the 284 sample log viral load results tested by the Roche AmpliCor version 1.5. This distribution is typical of the clinical samples received at the laboratory where 20% have viral load results <400 copies/ml (2.6 log) and 18% have viral load results >750 000 copies/ml (5.8 log).

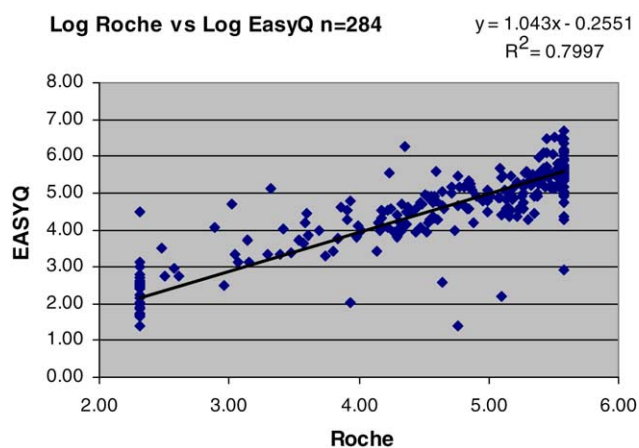


Fig. 2. The linear regression between EasyQ and Roche AmpliCor. Both axes are represented in log IU for both assays. The equation of the line and the R^2 -values are presented on the plot, which shows a significant linear relation between both assays and 79.9% of the data represented by the equation of the line.

3.2. Linear relationship

The Spearman's correlation coefficient $r = 0.874$, $p < 0.0001$ shows a significant correlation between the EasyQ assay (IU) and the Roche AmpliCor (IU) for this range of data ($n = 284$). The R^2 -value from the linear regression (in Fig. 2) illustrates that 79.9% of the data are represented by the equation of the line ($y = 1.043x - 0.2551$), with a significant slope ($p < 0.0001$). Since the Roche AmpliCor version 1.5 is limited to result reporting in the linear range (400–750 000 copies/ml), excluding these values from the linear regression does not modify the R^2 (0.713) or the significance of the slope ($p < 0.001$) from the equation of the line ($y = 0.77x + 0.993$). The Spearman's correlation coefficient also retains the significant linear relation ($r = 0.81$, $p < 0.001$) between the assays. This indicates that both assays retain the linear relationship over all log intervals ($n = 214$) and are not influenced by the end points in the equation of the line.

3.3. Method comparison

In order to determine agreement between both methods, the Bland–Altman model (Bland and Altman, 1986) was applied and represented in Fig. 3. This determined that the mean difference between the assays is log 0.0668 (CI –0.008, 0.142) and the limits of agreement (mean \pm 2 standard deviation) are 1.35 and –1.22. The small difference (bias) and narrow limits of agreement show good agreement between these two methods. At least nine (3.2% patients) outliers (points beyond the limits of agreement) are evident from Fig. 3, and are randomly dispersed throughout the log intervals. This highlights that the assays are not more or less problematic in any log interval. Evident from this plot, however, is that most of the outliers in the <4 log interval produce EasyQ values that read higher than the Roche AmpliCor. Conversely

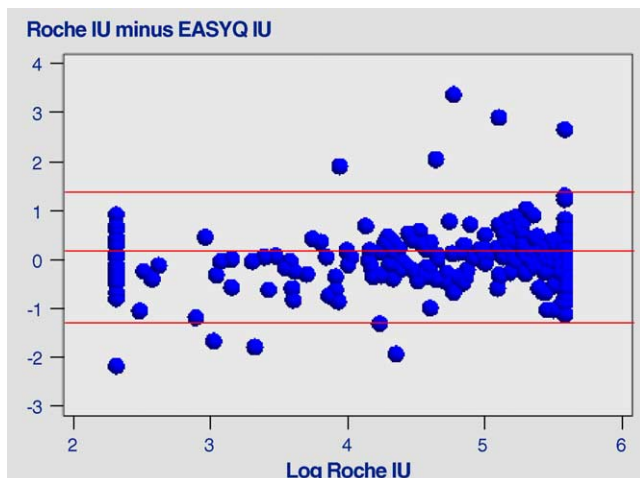


Fig. 3. Bland–Altman analysis to measure agreement between the two assays. The mean difference [0.068 log IU/ml (CI -0.008 , 0.142)] and limits of agreement (mean \pm 2 S.D.) (1.35 , -1.22) are indicated on the plot. The mean difference between the assays is represented on the y-axis against the log values of the Roche Amplicor in IU (taken as the standard in this analysis).

in the >4 log intervals, most of the outliers have Roche values that read higher than the EasyQ. This is similarly shown using the percentage similarity model (Scott et al., 2003) as described in Fig. 4. According to both models the Roche Amplicor (IU/ml) on average produced higher results than the EasyQ. The percentage similarity model shows overall good accuracy (percentage similarity $\mu = 98.7\%$) and precision (mean percentage difference = $1.3 \pm 9.3\%$) between the two methods with overall good agreement (percentage similarity CV = 9.4%) between the log IU/ml result reported.

3.4. Reproducibility of the EasyQ assay

Technologists performing the EasyQ do differ in reproducibility. Reproducibility repeated five times on one sample in the log 6 IU range yielded CV variance of 1.3 – 2.84% for two well-trained technologists. Similarly in the log 3 IU range, the variance remained stable for one technologist at 1.27% but increased to 4.4% for the second technologist. In addition, using the percentage similarity model, the technologist with the lowest CV on average produced higher results in both log intervals than the technologist with the poorer precision. This may explain the outliers in the lower log intervals as described above for method comparison, that greater variability can occur with the EasyQ assay in the lower log interval range.

3.5. Effect of specimen volume on the EasyQ assay

The volumes used for reproducing samples with the EasyQ assay ranged from 200 , 300 , 500 , $750 \mu\text{l}$ and the recommended volume $1000 \mu\text{l}$. A specimen in the log 6 IU range had a lower CV (2.2%) than a specimen in the lower log 4 IU

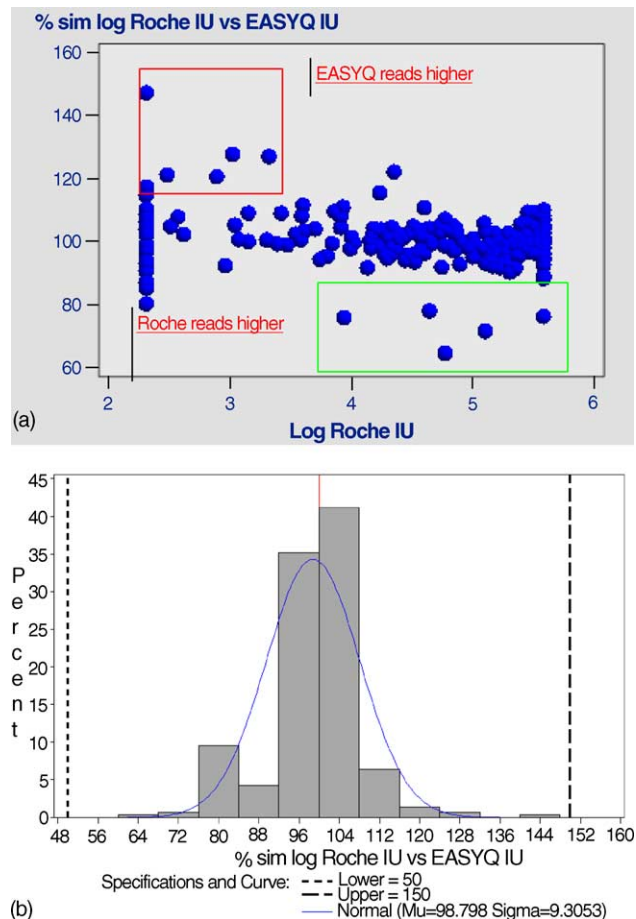


Fig. 4. The Percentage similarity model (Scott et al., 2003) for measuring agreement between the Roche and the EasyQ assays reported in IU/ml. (a) The scatter plot with percentage similarity on the vertical axis and the Roche log IU/ml values on the horizontal axis illustrates the outliers in boxes. The outliers (3.2% total patients) in the <4 log interval produces higher results on the EasyQ system and the outliers >4 log interval produce results that read higher on the Roche assay. (b) The percentage similarity values represented in a histogram format show good accuracy ($\mu = 98.7\%$) and precision (MPD = $1.3 \pm 9.3\%$) with overall good agreement (CV = 9.4%). The reference lines (dotted) are drawn at 50% , 150% and the 100% similarity line.

interval (3.2% CV). The variability increased with the lower log value.

3.6. Retrospective evaluation

A retrospective evaluation of the 102 runs conducted using the NASBA EasyQ System; each containing 24 samples, representing a total of 2448 samples was conducted. A negative and positive control was added to each run. An overall 182 (7.4%) samples were reported as inconclusive or invalid on the EasyQ system. Of these specimens 74 could be repeated (sufficient volume available for repeat), but a repeat invalid result was obtained in 40 samples representing 54% of the total repeats. Nine of the one hundred and two (8.8%) runs showed low-level contamination in the negative control (27 – 81 IU/ml). Total assay time when performed under

average laboratory operating conditions is approximately 3 h and 45 min for 24 samples. This is divided into the following stages: lysis preparation (40 min), binding (30 min), washing (30–40 min), elution (20 min); set up and run (25 min); time on analyzer (60 min) and resulting (10–20 min). If the lysis buffer is added at referral site, the total laboratory time is shortened by 40 min, resulting in a time to reporting of approximately 3 h.

4. Discussion

Currently few published studies exist on the performance of the NucliSens EasyQ assay version 1.1 from bioMérieux or its use in combination with the semi-automated NucliSens miniMag system extraction system. Little evaluation has been conducted to date in HIV-1 subtype C samples (Segondy et al., 2002). Recent work published from Israel by Gottesman et al. (2004), suggests an under-estimation of subtype C using the older version of NASBA, the NucliSens QT assay. Other studies have revealed quantitation problems with subtype A (Nkengasong et al., 1998). This evaluation, thus represents important data for individuals wishing to implement the assay in the context of HIV-1 subtype C (Papathanasopoulos et al., 2003; Scriba et al., 2001; van Harmelen et al., 2001).

Overall good correlation was demonstrated in the direct comparison of the samples using the NucliSens EasyQ assay versus the Roche Amplicor version 1.5 ($r=0.874$, $p<0.001$ $n=284$). The mean difference (bias) between these two methods [0.068 log IU/ml (CI -0.008 , 0.142)] is within acceptable limits as reported in previously published comparisons of commercially available viral load assays: (1) comparison of Roche Amplicor 1.5 version versus bDNA version 3.0: mean log difference of 0.274 log (Murphy et al., 2000), (2) standard Roche Amplicor Monitor versus NucliSens QT assay: mean difference of 0.109 log RNA copies/ml (Dyer et al., 1999), (3) NucliSens QT versus bDNA version 3.0: mean difference of 0.161 log RNA copies/ml (Murphy et al., 2000). Similarly, reproducibility in this study was good with standard deviations of 0.083 and 0.172 for the high copy number specimen and 0.049 and 0.164 for the low copy number being reported between technologists. The differences suggest the semi-automated extraction does lend itself to potential user differences. The suggestion would be that proficiency of each user is monitored regularly in this fashion within the laboratory. Sample input volume did appear to influence the results in the limited evaluation conducted in this study, which has been previously reported for the earlier NucliSens QT assay (Nowicki et al., 2001).

In this study, particular emphasis was placed on the technical ease with which the assay could be conducted. The assay was felt to be easy, but labour intensive at the level of the front-end manual extraction step. There is no walk away time during the extraction period. This may present difficulties when one is forced to scale up to meet the needs of a large ARV rollout program where high volume viral load testing

is a necessity. This issue should be resolved in future with the development of an automated extraction system based on bioMérieux's Boom technology which is due for release in South Africa in the fourth quarter of this year (Berghuis et al., 2004).

Another concern that was identified was the number of repeats/invalid results, which was an overall 7.4% of samples conducted. This is similar to findings reported in a study in Glasgow where 14/182 (7.6%) samples were invalid, 5.5% of these were at level of the amplification step and four samples could not be investigated due to "no eluate" failure with the extractor (Cameron, 2004). The second concern is the potential for low-level contamination with less experienced staff in high volume environments. In this study, contamination occurred at the level of both the extraction and amplification steps.

In conclusion, this assay represents a reasonable option for HIV viral load monitoring. Further assay validation is suggested including a longitudinal patient follow-up once the automated extractor becomes available towards the end of the year.

Acknowledgements

The National Health laboratory Service and NIH CIPRA Grant for funding.

References

- Berghuis, I., Kreuwel, H., van de Wiel, P.A., 2004. Evaluation of a new automated system for ultrasensitive HIV-1 viral load monitoring based on magnetic silica extraction and real-time NASBA technology. In: XV International AIDS Conference, 13–16 July.
- Bland, J.M., Altman, D.G., 1986. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1 (8476), 307–310.
- Cameron, S.p.c., 2004. Comparison of the NucliSens EasyQ HIV-1 viral load measurement with NucliSens HIV-1 QT and Roche Amplicor HIV-1 version 1.5 on routine clinical plasma specimens of HIV infected patients.
- Dyer, J.R., Pilcher, C.D., Shepard, R., Schock, J., Eron, J.J., Fiscus, S.A., 1999. Comparison of NucliSens and Roche Monitor assays for quantitation of levels of human immunodeficiency virus type 1 RNA in plasma. *J. Clin. Microbiol.* 37 (2), 447–449.
- Ginocchio, C.C., Tetali, S., Washburn, D., Zhang, F., Kaplan, M.H., 1999. Comparison of levels of human immunodeficiency virus type 1 RNA in plasma as measured by the NucliSens nucleic acid sequence-based amplification and Quantiplex branched-DNA assays. *J. Clin. Microbiol.* 37 (4), 1210–1212.
- Gottesman, B.S., Grosman, Z., Lorber, M., Levi, I., Shitrit, P., Mileguir, F., Gottesman, G., Chowers, M.Y., 2004. Measurement of HIV RNA in patients infected by subtype C by assays optimized for subtype B results in an underestimation of the viral load. *J. Med. Virol.* 73 (2), 167–171.
- http://www.iapac.org/text/pdf/WHO_2002_Guidelines_for_scaling_up_ARV_in_resource_limited_settings. WHO document, April.
- Murphy, D.G., Cote, L., Fauvel, M., Rene, P., Vincelette, J., 2000. Multicenter comparison of Roche COBAS AMPLICOR MONITOR version 1.5, Organon 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.* 38 (11), 4034–4041.
- Nkengasong, J.N., Kalou, M., Maurice, C., Bile, C., Borget, M.Y., Koblavi, S., Boateng, E., Sassan-Morokro, M., Anatole-Ehounou, E., Ghys, P., Greenberg, A.E., Wiktor, S.Z., 1998. Comparison of NucliSens and Amplicor monitor assays for quantification of human immunodeficiency virus type 1 (HIV-1) RNA in plasma of persons with HIV-1 subtype A infection in Abidjan, Cote d'Ivoire. *J. Clin. Microbiol.* 36 (9), 2495–2498.
- Nowicki, M.J., Benning, L., Bremer, J.W., Meyer III, W.A., Hanson, C., Brambilla, D., Silver, S., Kovacs, A., 2001. Longitudinal variability of human immunodeficiency virus type 1 RNA viral load measurements by nucleic acid sequence-based amplification and NucliSens assays in a large multicenter study. *J. Clin. Microbiol.* 39 (10), 3760–3763.
- Papathanasopoulos, M.A., Hunt, G.M., Tiemessen, C.T., 2003. Evolution and diversity of HIV-1 in Africa—a review. *Virus Genes* 26 (2), 151–163.
- Scott, L.E., Galpin, J.S., Glencross, D.K., 2003. Multiple method comparison: statistical model using percentage similarity. *Cytometry* 54B (1), 46–53.
- Scriba, T.J., Treurnicht, F.K., Zeier, M., Engelbrecht, S., van Rensburg, E.J., 2001. Characterization and phylogenetic analysis of South African HIV-1 subtype C accessory genes. *AIDS Res. Hum. Retroviruses* 17 (8), 775–781.
- Segondy, M., Montes, B., Soriano, V., Cuypers, H., Ferre, V., Koppelman, M., Mendosa, d., Oosterlaken, T.A., van de Wiel, P.A., 2002. Multicenter performance evaluation of Nuclisens EASYQ HIV-1. In: XIV International AIDS Conference, May.
- van Harmelen, J., Williamson, C., Kim, B., Morris, L., Carr, J., Karim, S.S., McCutchan, F., 2001. Characterization of full-length HIV type 1 subtype C sequences from South Africa. *AIDS Res. Hum. Retroviruses* 17 (16), 1527–1531.
- Weusten, J.J., Carpay, W.M., Oosterlaken, T.A., van Zuijlen, M.C., van de Wiel, P.A., 2002. Principles of quantitation of viral loads using nucleic acid sequence-based amplification in combination with homogeneous detection using molecular beacons. *Nucleic Acids Res.* 30 (6), e26.