Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

A New Affordable Flow Cytometry Based Method to Measure HIV-1 Viral Load

Burkhard Greve,[1*] Jürgen Weidner,[2] Uwe Cassens,[3] Georgina Odaibo,[4] David Olaleye,[4] Walter Sibrowski,[5] Doris Reichelt,[6] Ines Nasdala,[2] Wolfgang Göhde[7]

[1]Department of Radiotherapy-Radiooncology, University Hospital Münster, 48149 Münster, Germany

[2]Partec GmbH, Am Flughafen 13, 02828 Görlitz, Germany

[3]Institute of Transfusion Medicine, Laboratory Medicine and Medical Microbiology, Klinikum Dortmund gGmbH, 44137 Dortmund, Germany

[4]Department of Virology, College of Medicine, University of Ibadan, Ibadan, Nigeria

[5]Department of Transfusion Medicine, University Hospital Münster, Münster, Germany

[6]Department of Internal Medicine, University Hospital Münster, Münster, Germany

[7]Medical Faculty, University Hospital Münster, 48149 Münster, Germany

Received 17 March 2008; Revision Received 2 October 2008; Accepted 20 October 2008

Additional Supporting Information may be found in the online version of this article.

B. Greve and J. Weidner have contributed equally to this article.

*Correspondence to: Burkhard Greve, Ph.D., Department of Radiotherapy-Radiooncology/University Hospital Münster, Albert-Schweitzer-Strasse 33, D-48149 Münster, Germany

Email: greveb@uni-muenster.de

Published online 25 November 2008 in Wiley InterScience (www.interscience.wiley.com)

DOI: 10.1002/cyto.a.20676

ISAC
International Society for Advancement of Cytometry

Abstract

Most commercially available assays for diagnosis of HIV infection have shown shortcomings in the detection and quantification of rare genotypes of the virus. Most of the assays do not detect subtype O (outlier) and/or N (nonmajor, nonoutlier) or new circulating recombinant forms (CRFs), which are becoming more important in sub-Saharan Africa. Furthermore, the commonly available tests require costly measuring devices and expensive test kits, which are not easily affordable for developing countries. This study was designed to explore solutions to the problem of viral load assays in developing countries. Two forward primers, digoxygenin (DIG) and dinitrophenol (DNP) labeled, and one biotin (BIO) labeled reverse primer were used to amplify both, the HIV-1-5′LTR (long terminal repeat) region and an internal standard sequence. The two polymerase chain reaction (PCR)-products were captured by anti-DIG and anti-DNP antibody coated microparticles. Flow cytometric analyses were carried out after labeling with streptavidin-R-phycoerythrine. The primer system used recognized all HIV-1 subtypes. A coamplified internal standard warranted the functionality of the PCR and allows reproducible viral load measurements. Two drawbacks of current viral load measurements are overcome by the flow cytometry based test described hereof. First, all known worldwide relevant HIV-1 subtypes including subtypes O, N, and new CRFs are quantifiable with high sensitivity (50 to $>1 \times 10^6$ copies per PCR). Second, the cost per test can be reduced to less than 12 US$ instead of the current 50–100 US$. Additionally, the test described in this report offers the possibility to perform complete monitoring program (CD4 T-cell count, CD4% and viral load) for the first time, with the same device for HIV-infected persons.

Key terms

HIV; flow cytometry; viral load

THE treatment of HIV-infection by highly active antiretroviral therapy (HAART) requires regular monitoring of different blood parameters. Beside CD4 T-cell number (1–3), which is described as the best surrogate marker (4) or for children, the more significant CD4% value (1,5,6) blood serum virus concentration (viral load) represents the most meaningful parameter to evaluate treatment success and to decide about the initiation of HAART (7). Virus detection is also important for evaluation of prevention of mother-to-child transmission, chemoprophylaxis, and for testing newborns and mother's milk (8) or in persons with atypical course of disease (9). Usually, viral load tests based on highly specific real time polymerase chain reaction (PCR) allow the determination of viral serum concentration over a wide scale. Nevertheless, genetic variants of HIV-1 often impair the test results due to problems of primer mismatch.

In the developed western world, subtype B is the predominant strain of HIV-1, and commonly available tests are optimized for this subtype, but in Africa and Asia where more than 95% of the infected individuals reside there exist other subtypes and circulating recombinant forms (CRFs; 10,11). Some of these genetic variants like

O- and/or N-subtype or new CRFs are not detectable by the common tests, which lead to false or negative test results despite of clinical AIDS symptoms (Prof. Olaleye, personal information), a situation that results in insufficient therapy monitoring and suboptimal patient management. In addition to the limitation of quality, extremely high cost per test (50–100 US$) (12,13) and high costs for dedicated equipment that can only be used for such assays impedes the implementation of such tests and devices in developing countries.

Therefore, we explored alternative technical solutions to determine viral load in resource-limited countries. An optimal solution would be, if a single affordable device can be used to measure all therapy-relevant monitoring parameters [CD4 T-cell count, CD4 percentage of lymphocytes (CD4%), and viral load] (13). We achieved this objective by combining a standard PCR reaction with flow cytometric technology. After amplification of the 5′ LTR region of the HIV-1 genome with specifically labeled primers, the products are captured by microparticles and subsequently analyzed by flow cytometry. The copreparation and amplification of an internal standard allows quality control of all the steps from the purification of RNA over PCR-reaction to the quantification of amplified HIV-1 product. Flow cytometry is usable for all other follow-up relevant parameters like CD4 T-cell count or CD4 %.

Materials and Methods

Reference Panel

The first international reference panel for HIV-1 RNA genotypes from the National Institute of Biological Standards and Control (NIBSC) (NIBSC code: 01/466; WHO/BS/03.1961) was used to validate the new test procedure. The reference panel consisted of 11 coded samples representing different HIV-1 genotypes (subtypes A, B, C, D, AE, F, G, AG-GH, groups N and O) as well as a negative diluent control. Additionally, subtypes K, J, CRFO2AG, CRF B/G, CRF11cpx, and three further O subtypes (Reference No NIBSC: ARP 191, 194, 168) that are highly distributed in sub-Saharan countries were tested. Two other B subtypes (Reference No NIBSC: ARP 101, 129) that were not contained in the reference panel were also tested. The viral load of these samples was determined by Roche Amplicor HIV-1 Monitor Test (version 1.5).

Oligonucleotide Design

The primer design was based on the publications of Holmes, Cleland, Rouet, Drosten, and Somogiy, who described two highly conservative regions in the 5′ LTR region of the HIV genome (8,14–17). In contrast to common real time PCR-tests (RT-PCR), the test described does not need a third conservative region for the detection probe (TaqMan, Scorpions, Molecular Beacon, etc.).

The test described herein is based on three primers, which enables the amplification of both, an internal control sequence and the corresponding virus sequences. One forward primer, specific for the internal control, is modified with dinitrophenol (DNP) at the 5′ end, while the other forward primer, specific for the HIV genome is modified with digoxygenin (DIG). The reverse primer binds at both, the internal control and the virus sequences, and is modified at the 5′ end with biotin (BIO). Amplified control- and HIV-sequences are captured separately by their DIG and DNP label using microparticles of different size (6.2 μm and 3.7 μm), coated with anti DIG and anti DNP antibodies. Phycoerythrine (PE)-conjugated streptavidine binds the PCR products by their biotin label and allows the specific detection and quantification by flow cytometry.

Internal Control (Artificial HIV-1 Standard)

A fragment of HIV-1 independent DNA sequence was amplified with a specific forward primer and a specific reverse primer, which was elongated with the complementary sequence of the HIV-1 specific 5′ LTR reverse primer (Fig. 1A).

After amplification, the PCR fragment was separated by gel electrophoresis and extracted with a Qiaquick gel extraction Kit (Qiagen, Hilden, Germany). The purified fragment was ligated with the TOPO TA Cloning Kit into a pCR II-TOPO vector (Invitrogen, Karlsruhe, Germany) and cloned into the *E. coli* strain TOP10. After transformation, the plasmids were isolated with the Qiaprep 8 Turbo Miniprep Kit (Qiagen) followed by EcoR1 digestion to control the length of the insertion fragment. To avoid carryover of genomic DNA, the plasmid was purified by gel electrophoresis and quantified spectrophotometrically at 260/280 nm.

Afterwards, the plasmid DNA was transcribed into RNA with the RiboMax Large scale RNA production Systems-SP6 and T7 (Promega, Mannheim, Germany) as recommended by the manufacturer. After DNA digestion, the RNA was purified with NAP10 columns and quantified spectrophotometrically.

The transcribed RNA was used as an internal standard during the HIV-RNA isolation, reverse transcription, and PCR amplification, respectively. Furthermore, the DNA plasmid with the same sequence was the basis for standard curves with $1 \times 10^2 - 1 \times 10^6$ copies at the start of the PCR amplification. The internal control did not influence the efficiency of the virus PCR, as revealed by gel electrophoresis (Fig. 1B).

HIV-1 RNA Isolation

Viral RNA was isolated from plasma samples by using TRIZOL, LS reagent (Invitrogen, Karlsruhe, Germany), but with the following changes. After mixing 400 μl of blood plasma with 1.2 ml TRIZOL, bromochloropropane (18) was used instead of chloroform to reduce contamination with human genomic DNA. Additionally, 50 μg/ml glycogen was added during the precipitation step to improve the amount of isolated RNA (19). All other steps were performed as recommended by the manufacturer.

Reverse Transcription

Reverse transcription was done according to the manufacturers instruction by using Superscript III First Strand Synthesis System (Invitrogen, Germany). Eight microliters of the RNA previously isolated was reverse transcribed with random hexamers. cDNA was stored at -20° until use or used for the PCR immediately after isolation.

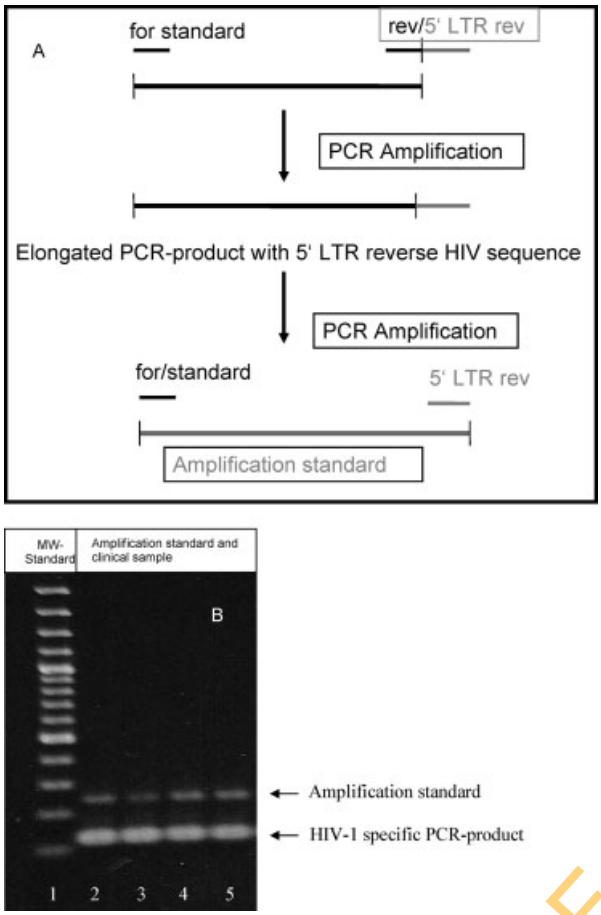

Figure 1. (**A,B**). Preparation strategy for an HIV-1 independent internal RNA standard sequence (A). One forward primer specific for the standard sequence and one reverse primer complementary to the standard sequence but elongated by a HIV-1 5′LTR specific sequence was used to amplify the standard. The resulting sequence is elongated by the HIV-1 5′ LTR specific reverse primer sequence. This allows both, the amplification of the HIV-1 product and the standard by the use of three primers (standard-for, HIV-1-for, HIV-1-rev). By this strategy, the used standard does not compete with the HIV-1 sequence for primer annealing (B) (Lane 1: 100 BP ladder; Lanes 2 and 3: 1,000 copies; and Lanes 4 and 5: 2,000 copies of the standard sequence amplified together with a constant HIV-1 concentration).

PCR

To avoid inadvertent carryover of former-amplified fragments, dUTPs instead of dATPs were used in any PCR amplification and uracil-DNA glycosylase (UDG) to digest possible cross contaminations (20). The reaction was carried out at 37°C for 15 min before PCR initiation. The PCR reaction master mix included the following components for each 25 μl reaction: 2.5 μl 10× buffer; 0.5 μl of the dNTP mix including 5 mM dUTPs, 5 mM dTTPs, 10 mM dATPs, dGTPs, dCTPs, 0.5 μl UDG 1 U/μl (Bioron, Ludwigshafen, Germany); 0.1 μl SuperTaq 5 U/μl (HT Biotechnology, Cambridge, UK); 0.1 μl Taq Antibody 1.1 μg/μl (Clontech, Heidelberg, Germany); 5 pmol target specific DIG labeled forward primer; 5 pmol internal control specific DNP-labeled forward primer and 5 pmol BIO-labeled reverse primer (Eurogentec, Genéve, Belgium); 2 μl cDNA (included 200 copies of the RNA standard) that was taken through the entire sample preparation procedure. The reaction tubes were cycled in an Eppendorf mastercycler using the following protocol: pretreatment at 95°C for 10 min, inactivation of the UDG, 28 cycles: 95°C for 30 s, 54°C for 30 s, 72°C for 30 s, and posttreatment 72°C for 4 min. The real-time measurements were done under the same conditions on a RotorGen 6000 HRM (Corbet Research, Sidney, Australia). For amplification, SYBR Green PCR Core Reagents (Applied Biosystems, Foster City) was used.

Purification of the PCR Product

To remove unused primers, 20 μl of the PCR product was washed in 60 μl of 8 M sodium perchlorate and 4 μl of MagPrep silica beads (Promega). The samples were incubated for 10 min at 50°C. Afterwards, the amplification products were separated with a magnetic separator (Promega). The supernatant was discarded, and the sample was resuspended in 20 μl H_2O. Finally, the sample was incubated for 5 min at 50°C and again magnetically separated. The purified PCR product that was used for the beads binding procedure was located in the supernatant.

Beads Binding

One microliter of microbeads (13,500 per μl) was activated by the addition of 50 μl of binding buffer (150 mM NaCl, 15 mM Tris/HCl pH 7.5) and centrifuged at 16,000 rcf (*g*) for 2 min. By discarding the supernatant, the activated microbeads were ready for the following binding procedure.

One microliter of the PCR product was incubated with 1 μl anti DIG beads (6.2 μm), respectively, and 1 μl anti DNP beads (3.7 μm) (Partec, Münster, Germany). Fluorescent labeling was performed by the addition of 1 μl strepdavidine-R-PE (Qiagen, Germany) to the reaction mixture (Fig. 2). The samples were incubated on a turning incubator for 15 min at room temperature and finally filled up with 1 ml TE buffer for flow cytometric measurement.

Flow Cytometry

Flow cytometric measurements were conducted using a PAS III flow cytometer (Partec, Germany) equipped with a 488 nm argon laser. The size-specific differentiation of the measured microparticles (3.7 and 6.2 μm) was performed by FCS/SSC plot, and a gate was set on each population. PE-specific fluorescence was measured in fluorescence 2 (FL2 with a 590 nm bandpass filter). Ten thousand events were collected, and the mean fluorescence intensity was calculated by setting a range over the whole measuring scale (0–1,000) in FL2 (log 4) separately for each microbead population (Supporting Figure 1).

Statistical Analysis

Results of repeated measurements (fivefolds) of different standard fragment concentrations were used to calculate the

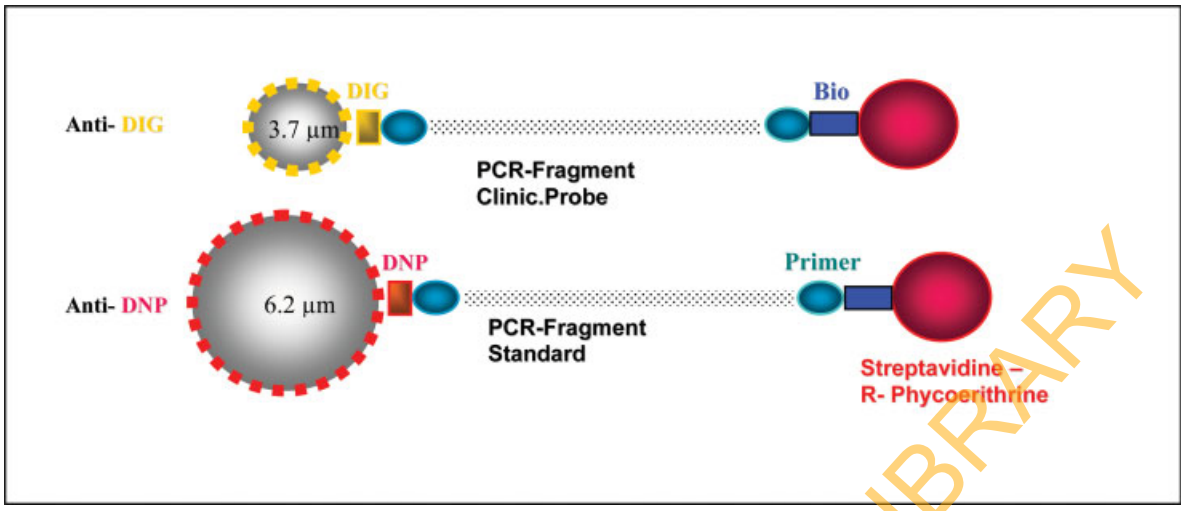

Figure 2. Schematic picture of capturing PCR-products by microparticles. The DIG-labeled PCR-product of the clinical probe is captured specifically by anti-DIG labeled 3.7 μm beads and the standard PCR-fragment by anti-DNP labeled 6.2-μm beads. Phycoerythrine-labeled streptavidine binds to the biotine-labeled 5′ end of the PCR-fragment.

mean value and the standard deviation of the flow cytometric measurement for each measuring point. Linear regression was calculated for the real-time PCR analysis by comparing standard fragment copy number with threshold cycle number (CT). *P*-values were calculated by using the *t*-test to verify the lower limit of detection ($P \leq 0.05$ differences are significant).

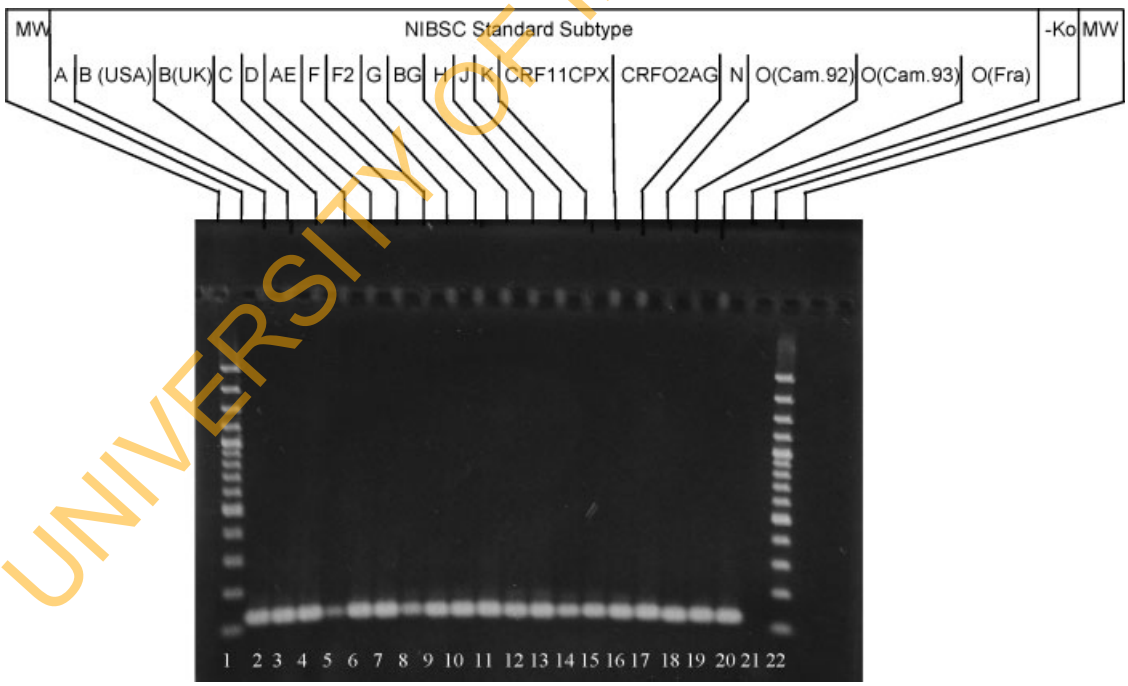

Figure 3. The NIBSC standard and the additional amplified subtypes were used to validate the specificity of the here-described new primer system. The amplification products were separated on a 2% (w/v) agarose-gel and stained with ethidium bromide. All subtypes were amplified with the same high specificity. Differences in fluorescence intensity resulted from varied concentration of single subtypes. (Lane 1: 100 BP ladder (MW), Lane 2: subtype A, Lane 3: subtype B (USA), Lane 4: subtype B (UK), Lane 5: subtype C, Lane 6: subtype D, Lane 7: subtype AE, Lane 8: subtype F, Lane 9: subtype F2, Lane 10: subtype G, Lane 11: subtype BG, Lane 12: subtype H, Lane 13: subtype J, Lane 14: subtype K, Lane 15: CRF 11 cpx, Lane 16: CRF 02 AG, Lane 17: subtype N, Lane 18: subtype O Cameroon 1992, Lane 19: subtype O Cameroon 1993, Lane 20: subtype O France 1994, Lane 21: negative control (−Ko), Lane 22: 100 BP ladder (MW).

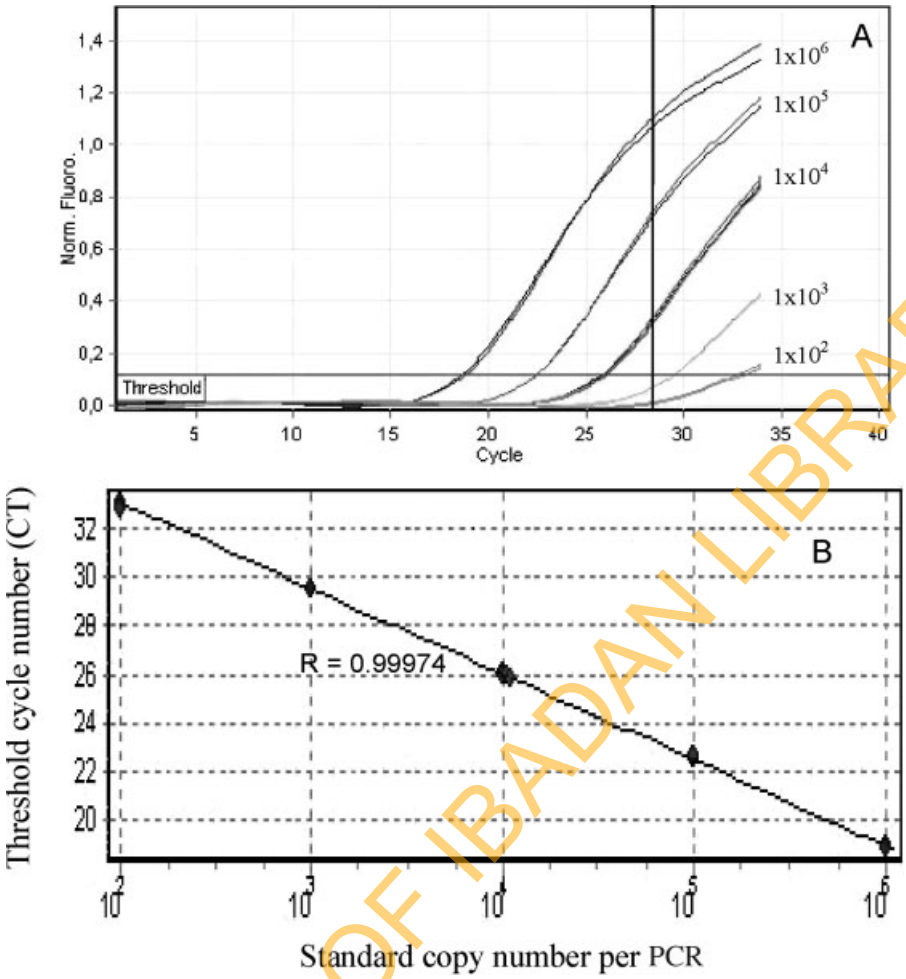

Figure 4. (**A,B**). Known concentrations (1×10^2 to 1×10^6 per PCR) of the internal standard was amplified by real-time PCR. Development of fluorescence (A) and the corresponding standard curve (B) is given. The standard curve shows the number of cycles for leaving the threshold (CT) compared with the copy number of the standard. The vertical line shows the cycle number (28 cycles), after which the products were used for flow cytometric determination.

Results

A new viral load test based on flow cytometric measurement was established and evaluated concerning its specificity, sensitivity (limit of detection), and reliability. The specificity was investigated by the verification of all known subtypes using a reference panel (NIBSC 01/466) plus additional subtypes and CRFs ordered for the study. The sensitivity was specified by serial dilution of an internal standard, and the reliability was determined by repeated measurement of different internal standard concentrations. The internal standard with an HIV-1 5′LTR specific sequence ensured the correct course of purification and amplification of the virus and allowed the determination of viral load.

Determination of Specificity

A reference panel from the NIBSC (Order No.01/466) and the additionally ordered subtypes and CRFs obtained as indicated later were used to determine the specificity of the selected PCR primers. Figure 3 shows the amplified PCR products of all the available subtypes (lanes 2–20) and a control without virus (lane 21) on a agarose gel. Lanes 2–20 represent the following subtypes: A, B (USA), B (UK), C, D, AE, F, F2, G, B/G, H, J, K, CRF11cpx, CRF02AG, N, O (Cameroon 1992), O (Cameroon 1993), O (France 1994). The test was repeated thrice. All the subtypes were amplified while the negative control showed no product. The differences in the staining intensity resulted from the different virus concentrations of each panel strain. The same differences in concentration were analyzed by quantitative real time PCR (data not shown).

Determination of Sensitivity

Standard DNA sequences (100; 1,000; 10,000; 100,000; 1,000,000 copies per PCR) were used to determine the lower limit of detection, the measuring range of the real-time PCR, and the optimal cycle number for the flow cytometric analysis. With each PCR-cycle, the fluorescence intensity increases with increasing copy numbers of standard and exceeded a threshold at dedicated cycle numbers (CT) (Fig. 4A). Comparing the

virus copy number with the cycle number of leaving the threshold, the whole range of dilutions resulted in a high linear regression ($r = 0.99974$) (Fig. 4B). For flow cytometric determination, 28 PCR-cycles were determined to be optimal to quantify the range of 50–1,000,000 virus particles (vertical line in Fig. 4A).

The flow cytometric measuring principle is shown in Figure 5A. Analysis of known concentrations (50 to 1×10^6 copies per PCR) of the standard resulted in a sigmoidal standard curve when plotting the copy number (per PCR) and the mean fluorescence, respectively (Fig. 5B).

The reproducibility of the flow cytometric viral load test was determined by repeated (five times) measurement of the aforementioned dilutions. The mean values and standard deviations were determined as shown in Table 1. There were significant differences in fluorescence at lower copy numbers (0 and 50 copies per PCR; $P = 0.0144$; 50 and 100 copies per PCR; $P = < 0.001$; 100 and 500 copies per PCR; $P = < 0.001$).

Blood samples of HIV-infected ($n = 20$) and noninfected ($n = 20$) individuals were analyzed for viral load using the new flow cytometric test and the standard real-time PCR assay. Both test systems revealed similar viral load results of the HIV-infected samples, which are shown in Table 2. In all 20 samples of the uninfected individuals, the virus was not detectable. A viral load standard from the Paul Ehrlich Institut (PEI) with a given virus copy number of 4.90 log 10 per ml (±0.5 log 10) was quantified thrice. The results of both measuring methods reside in the aforementioned given range of the PEI standard (Table 2).

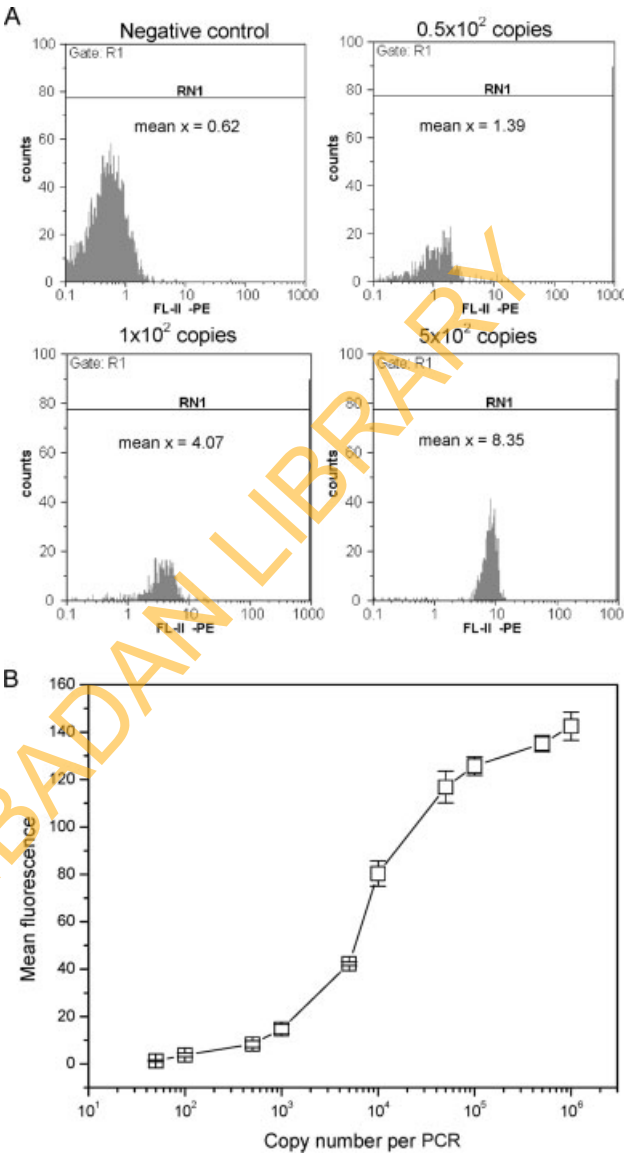

Figure 5. (**A,B**). Known concentrations (50 to 1×10^6 per PCR) of the internal standard were used to validate the flow cytometry based viral load test. The PCR reaction was stopped after 28 cycles, and the products were captured by microbeads. The fluorescent labeling was performed by phycoerythrine-conjugated streptavidine. Examples of the flow cytometric analysis with 50 to 5×10^2 copies, and 1×10^6 copies of the internal standard are given in A. The mean fluorescence corresponds to the given mean *x* value. The standard curve over the whole measuring range is shown in B.

Discussion

Accurate determination of viral load is of crucial clinical importance for patients infected with HIV or suffering from AIDS (21). Presently, three methods are approved by the Food and Drug Administration, these are HIV-RNA RT-PCR assay (Amplicor HIV-1 Monitor, version 1.5; Roche Molecular Systems), the branched-chain assay (bDNA; Versant HIV RNA 3.0 Assay; Bayer Diagnostics), and the nucleic acid sequence-based amplification (NucliSens HIV QT; bioMérieux) (22,23), but all these tests are optimized for subtype B, which is the common strain in resource-rich settings. Globally non-B subtypes like A/E, C, O, N, or new CRFs predominate (24) and raises the question whether the aforementioned tests are useful in those countries. In fact, these tests are very expensive (50–100 US$) (12) and susceptible, and costly equipment is necessary for the measurement and analysis. Therefore, several newly developed PCR assays have been published, which are designed for developing countries, essentially to recognize the whole panel of HIV-1 subtypes at reduced costs (8). Although the disadvantage of specificity is overcome by these tests, the same susceptible and costly equipment is necessary like ultracentrifuge and real-time PCR. Both are not commensurably available standard laboratory equipment in developing countries and are definitely not useable in rural areas.

Over the past few years, flow cytometric devices of different suppliers have been used in numerous African and Asian countries to assess CD4 T-cells of HIV-infected people (25). Some of these devices are robust, independent of external power supply and usable under mobile conditions (26). Insofar, this technical infrastructure would be well suited for viral load determination. The problem was to quantify PCR products by flow cytometry, and this has been overcome by coupling fluorescence-labeled amplification products to microbeads (27). Microsphere-based flow cytometric analyses become more and more important in molecular analysis, and multiplexed particle based immunoassays are now common place

Table 1. Reproducibility of measurement

PLASMID CONCENTRATION PER PCR	0	50	100	500	1×10^3	5×10^3	1×10^4	5×10^4	1×10^5	5×10^5	1×10^6
Fluorescence	0.59	0.85	3.26	9.72	17.67	42.11	81.31	111.19	122.02	134.41	147.71
	0.62	1.65	4.07	9.99	13.87	41.23	78.91	127.88	125.98	132.96	142.80
	0.54	0.97	4.90	8.35	12.71	43.65	83.79	129.05	133.12	137.37	138.73
	0.66	1.39	4.15	6.62	16.59	42.81	86.50	121.27	123.74	130.71	134.13
	0.77	1.62	2.57	7.03	13.06	42.34	71.01	113.97	123.68	140.58	133.05
Mean value	0.64	1.30	3.79	8.34	14.78	42.43	80.30	120.67	125.70	135.20	139.28
Standard deviation	0.09	0.36	0.89	1.52	2.22	0.89	5.91	8.02	4.37	3.85	6.10

Flow cytometric determined fluorescence values of five independent experiments.

(28). However, in the present study we used a multiplex particle system to quantify PCR-products of both, an internal standard sequence and a HIV-specific sequence in parallel.

According to some recent publications, we used the LTR region of the HIV-1 genome to amplify all subtypes with the same high specificity (8,14–17) and were able to verify this by the amplification of a NIBSC reference panel. Dilution experiments revealed a reliable measuring range of 50 to 1×10^6 virus particles per PCR and a limit of detection of 50 virus particles per PCR. Differences in fluorescence intensity at lower copy numbers (50, 100, and 500 copies per PCR) were statistically significant.

The NIH recommended the initiation of treatment for all patients who have less than 350 CD4 T-cells or viral load over 1×10^5 virus particles per ml (29); thus, this flow cytometric measuring range does comply with the requirements for treatment decision. The detection limit can be enhanced by decreasing the microbeads concentration; thus, this test is also usable for treatment follow-up and the quality control of blood products. In this context, it is important to verify the accuracy of the here described test. This is warranted by an internal control that is amplified despite of missing virus and thus guarantees the routine use of this test with high sensitivity and accuracy over a broad virus concentration. Accuracy is also underlined by the test of blood samples of HIV-infected ($n = 20$) and noninfected ($n = 20$) individuals. The flow cytometric test recognized the infected samples in all cases and viral load results showed a good correlation with that of the standard real-time PCR assay. All samples of noninfected individuals were detected as HIV-negative, and in these cases, both tests were unable to amplify virus sequences.

Table 2. Measurement of blood samples from HIV-infected individuals (n = 20) by flow cytometry and standard real-time PCR (values expressed as log 10)

SAMPLE NO	FLOW CYTOMETRY	STANDARD REAL-TIME PCR
1	5.36	5.59
2	5.29	5.52
3	5.55	5.50
4	6.67	6.14
5	6.03	6.13
6	5.62	5.71
7	5.27	5.59
8	5.48	5.51
9	5.65	5.89
10	4.43	4.49
11	5.71	5.89
12	5.79	5.84
13	5.48	5.54
14	5.24	5.43
15	5.66	5.76
16	6.10	6.25
17	2.96	3.26
18	5.96	6.07
19	5.41	5.52
20	4.51	4.88
PEI standard	5.20	5.27

PEI standard at a given concentration: 4.90 (±0.5 log 10).

The overall cost per test are less than 12 US$ and, thus, are far below current prices. The existing flow cytometric infrastucture for CD4 T-cell count is usable for the viral load test described herein and for new implementations the equipment costs reduced to 25,000 US$ for a flow cytometer and about 7,500 US$ for a standard PCR cycler.

In conclusion, we have developed a new PCR test that detects all known HIV-1 subtypes and applied this test for viral load determination on a common flow cytometer. By this, we could reduce the overall costs per test to less than 12 US$ and can use the available widespread flow cytometric infrastucture.

One future aspect of the described flow cytometry based viral-load test is to identify each individual HIV-1 subtype by the addition of the subtype-specific LNA-beacons. Thus, this would improve the individual treatment and would allow a better statistical allocation of the global virus subtype distribution.

Acknowledgments

The authors thank Annette van Dülmen, Steffi Kettelhut, and Angelika Vollmer for excellent technical assistance.

Literature Cited

1. Cassens U, Göhde W, Kuling G, Groning A, Schlenke P, Lehman LG, Traore Y, Servais J, Henin Y, Reichelt D, Greve B. Simplified volumetric flow cytometry allows feasible and accurate determination of CD4 T lymphocytes in immunodeficient patients worldwide. Antivir Ther 2004;9:395–405.
2. Greve B, Cassens U, Westerberg C, Göhde Jun W, Sibrowski W, Reichelt D, Göhde W. A new no-lyse, no-wash flow-cytometric method for the determination of CD4 T cells in blood samples. Transfus Med Hematother 2003;30:8–13.

3. O'Gorman MR, Zijenah LS. CD4 T cell measurements in the management of antiretroviral therapy—A review with an emphasis on pediatric HIV-infected patients. Cytometry Part B 2008;74B:19–26.
4. Bold A, Wurth R, Keller T, Trahorsch U, Voigt P, Schubert S, Sack U. Low-cost enumeration of CD4+ T cells using a density-based negative selection method (RosetteSep) for the monitoring of HIV-infected individuals in non-OECD countries. Cytometry Part A 2008;73A:28–35.
5. Greve B, Cassens U, Baumann C, Sibrowski W, Kuling G, Szabados F, Schlenke P, Reichelt D, Gebauer W, Göhde W. Determination of CD4 lymphocyte percentages using a new simplified and affordable no lyse flow cytometric protocol: A pilot study. J Lab Med (in press).
6. Ymeti A, Li X, Lunter B, Breukers C, Tibbe AG, Terstappen LW, Greve J. A single platform image cytometer for resource-poor settings to monitor disease progression in HIV infection. Cytometry Part A 2007;71A:132–142.
7. Rouet F, Rouzioux C. The measurement of HIV-1 viral load in resource-limited settings: How and where? Clin Lab 2007;53:135–148.
8. Drosten C, Panning M, Drexler JF, Hansel F, Pedroso C, Yeats J, de Souza Luna LK, Samuel M, Liedigk B, Lippert U, Sturmer M, Doerr HW, Brites C, Preiser W. Ultrasensitive monitoring of HIV-1 viral load by a low-cost real-time reverse transcription-PCR assay with internal control for the 5′ long terminal repeat domain. Clin Chem 2006;52:1258–1266.
9. Goicoechea M, Haubrich R. CD4 lymphoctye percentage versus absolute CD4 lymphocyte count in predicting HIV disease progression: An old debate revisited. J Infect Dis 2005;192:945–947.
10. Ivers LC, Kendrick D, Doucette K. Efficacy of antiretroviral therapy programs in resource-poor settings: A meta-analysis of the published literature. Clin Infect Dis 2005;41:217–224.
11. Kim JE, Beckthold B, Chen Z, Mihowich J, Malloch L, Gill MJ. Short communication: Identification of a novel HIV type 1 subtype H/J recombinant in Canada with discordant HIV viral load (RNA) values in three different commercial assays. AIDS Res Hum Retrovirus 2007;23:1309–1313.
12. Fiscus SA, Cheng B, Crowe SM, Demeter L, Jennings C, Miller V, Respess R, Stevens W. HIV-1 viral load assays for resource-limited settings. PLoS Med 2006;3:e417.
13. Abayomi EA, Landis RC. Flow cytometry as the spearhead for delivering sustainable and versatile laboratory services to HIV-burdened health care systems of the developing world: A Caribbean model. Cytometry Part B Clin Cytom 2008;74B:80–89.
14. Rouet F, Chaix ML, Nerrienet E, Ngo-Giang-Huong N, Plantier JC, Burgard M, Peeters M, Damond F, Ekouevi DK, Msellati P, Ferradini L, Rukobo S, Marechal V, Schrachsa N, Wakrim L, Rafalimanana C, Rakotoambinina B, Viard JP, Seigneurin JM, Rouzioux C. Impact of HIV-1 genetic diversity on plasma HIV-1 RNA quantification: Usefulness of the Agence Nationale de Recherches sur le SIDA second-generation long terminal repeat-based real-time reverse transcriptase polymerase chain reaction test. J Acquir Immune Defic Syndr 2007;45:380–388.
15. Cleland A, Davis C, Adams N, Lycett C, Jarvis LM, Holmes H, Simmonds P. Development of multiplexed nucleic acid testing for human immunodeficiency virus type 1 and hepatitis C virus. Vox Sang 2001;81:93–101.
16. Holmes H, Davis C, Heath A, Hewlett I, Lelie N. An international collaborative study to establish the 1st international standard for HIV-1 RNA for use in nucleic acid-based techniques. J Virol Methods 2001;92:141–150.
17. Somogiy S. Divergente Transkriptionskontrolle von HIV-1: Strukturelle und funktionale Analyse des LTR-Promotors der Gruppen M, N und O. Dissertation FU Berlin, 2002.
18. Chomczynski P, Mackey K. Substitution of chloroform by bromo-chloropropane in the single-step method of RNA isolation. Anal Biochem 1995;225:163–164.
19. Hengen PN. Carriers for precipitating nucleic acids. Trends Biochem Sci 1996;21: 224–225.
20. Pang J, Modlin J, Yolken R. Use of modified nucleotides and uracil-DNA glycosylase (UNG) for the control of contamination in the PCR-based amplification of RNA. Mol Cell Probes 1992;6:251–256.
21. Relucio K, Holodniy M. HIV-1 RNA and viral load. Clin Lab Med 2002;22:593–610.
22. Berger A, Scherzed L, Sturmer M, Preiser W, Doerr HW, Rabenau HF. Comparative evaluation of the cobas amplicor HIV-1 monitor ultrasensitive test, the new cobas ampliprep/cobas amplicor HIV-1 monitor ultrasensitive test and the versant HIV RNA 3.0 assays for quantitation of HIV-1 RNA in plasma samples. J Clin Virol 2005;33:43–51.
23. Holodniy M. HIV-1 load quantitation: A 17-year perspective. J Infect Dis 2006;194 (Suppl 1):S38–S44.
24. Kantor R. Impact of HIV-1 pol diversity on drug resistance and its clinical implications. Curr Opin Infect Dis 2006;19:594–606.
25. Janossy G, Shapiro H. Overview: Simplified cytometry for routine monitoring of infectious diseases. Cytometry Part B Clin Cytom 2008;74B:6–10.
26. Zijenah LS, Kadzirange G, Madzime S, Borok M, Mudiwa C, Tobaiwa O, Mucheche M, Rusakaniko S, Katzenstein DA. Affordable flow cytometry for enumeration of absolute CD4+ T-lymphocytes to identify subtype C HIV-1 infected adults requiring antiretroviral therapy (ART) and monitoring response to ART in a resource-limited setting. J Transl Med 2006;4:33.
27. Wedemeyer N, Pascher E, Cassens U, Gohde W. Quantification of PCR products using microparticles and flow cytometry. Ann Biol Clin (Paris) 2004;62:65–71.
28. Nolan JP, Mandy F. Multiplexed and microparticle-based analyses: Quantitative tools for the large-scale analysis of biological systems. Cytometry Part A 2006;69A: 318–325.
29. Council OoARA. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Available at http://aidsinfo.nih.gov/contentfiles/AdultandAdolescentGL.pdf 2006.