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Protocols

An improved PCR method for detection of HIV-1 proviral DNA of a wide range of subtypes and recombinant forms circulating globally

Jürgen Weidner[a,*], Uwe Cassens[b], Wolfgang Göhde[c], Walter Sibrowski[d], Georgina Odaibo[e], David Olaleye[e], Doris Reichelt[f], Burkhard Greve[g]

[a] Partec GmbH, Am Flughafen 13, 02828 Görlitz, Germany
[b] Institute of Transfusion Medicine, Laboratory Medicine and Medical Microbiology, Klinikum Dortmund gGmbH, Alexanderstraße 6-10, 44137 Dortmund, Germany
[c] Medical Faculty, University Hospital Münster, Domagkstraße 3, 48149 Münster, Germany
[d] Department of Transfusion Medicine, University Hospital Münster, Albert-Schweitzer-Straße 33, 48149 Münster, Germany
[e] Department of Virology, College of Medicine, University of Ibadan, Ibadan, Nigeria
[f] Department of Internal Medicine, University Hospital Münster, Germany
[g] Department of Radiotherapy-Radiooncology, University Hospital Münster, Albert-Schweitzer-Straße 33, 48149 Münster, Germany

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ABSTRACT

Proviral DNAs are being measured increasingly as a marker of the efficacy of highly active anti-retroviral therapy (HAART) and is accepted for the early diagnosis of perinatal HIV-1 infections. This requires a standardized test which enables the detection of a wide range of subtypes worldwide including O, N and circulating recombinant forms (CRFs). Based on a previous publication, a PCR – Test for HIV-1 provirus detection in peripheral blood mononuclear cells (PBMCs) was developed. Blood samples from 80 individuals infected with HIV-1 and 20 persons negative for HIV-1&2 from Africa and Germany were tested for the presence of HIV-1 provirus DNA. The primer system used enables the detection of proviral DNA despite the high concentrations of human DNA. The limit of detection was determined to be 5 copies per 10^5 cells. All 20 samples from persons negative for HIV were negative for HIV-1 proviral DNA while provirus DNA was amplified from 76 of the 80 (95%) samples from persons infected with HIV. The amplified products were detected by gel-electrophoresis, flow cytometry and real-time PCR. All three detection systems provided the same results.

1. Introduction

Infection with HIV-1 is characterized by the detection of HIV-1 plasma RNA, which in addition to the member of $CD4^+$ T-cells serves as the principal monitoring parameter for assessing disease progression and response to anti-retroviral therapy (**Cassens et al., 2004; Greve et al., 2003, 2008; O'Gorman and Zijenah, 2008; Zijenah et al., 2006**). Several well designed PCR-based tests are able to detect and quantify HIV-1 RNA in plasma samples of infected individuals (**Drosten et al., 2005, 2006; Rouet et al., 2007; Somogiy, 2002**). In contrast, HIV-1 proviral DNA is immobilized in peripheral blood mononuclear cells (PBMCs) and lymphoid tissues, which is highly resistant to HAART and serves as an infection reservoir. The determination of proviral HIV-1 DNA offers several significant information including: (1) early diagnosis of mother-to-child transmission (**Cassol et al., 1994**), (2) detection of viral sequences in the pre-seroconversion window period (**Moroney et al., 2006**), (3) the recognition if the HIV-1 RNA levels fall below the detectable limit during HAART (**Gibellini et al., 2004; Zhao et al., 2002**). Additionally, the detection of provirus DNA could be a useful marker for examining viral reservoirs and to assess the long-term impact of treatment (**Chun et al., 1998; Drosten et al., 2005; Finzi et al., 1997**).

A major problem in the amplification and detection of HIV proviral DNA is the presence of high amount of human DNA in the sample which influences the PCR. Furthermore, the small sample volumes of less than 200–500 μl blood caused by the difficulties encountered during veinpuncture in infants less than 18 months (**Piwowar-Manning et al., 2008**) makes great demands on the test.

Currently, the only commercial assay for the detection of HIV-1 DNA proviral is the Roche Amplicor HIV-1 DNA test which utilizes a nucleic acid hybridization system for post-amplification detection. The disadvantages of this test are recognised from several studies and the main drawback is the focus on HIV-1 subtype B which is predominant in Western Europe and North America; some HIV-1 subtypes and CRFs from Asia and Africa are not detectable (**Barlow et al., 1997; Bobkova et al., 2004; Bogh et al., 2000; Cunningham**

* Corresponding author at: University of Muenster, Pottkamp 2, 48149 Münster. Tel.: +49 251 8355389; fax: +49 251 8343852.
E-mail address: weidnej@uni-muenster.de (J. Weidner).

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et al., 2003; Gueudin et al., 2008; Moroney et al., 2006; Yun et al., 2002).

In this context an ideal PCR-based test should be sufficiently sensitive to detect all circulating HIV-1 subtypes and CRFs and should be applicable world wide at a price which is affordable in resource poor countries. It was possible to overcome these problems by adapting the primers used in a viral load test described previously (Greve et al., 2009) for the amplification and detection of proviral DNA in human blood samples.

2. Materials and methods

2.1. Subtype detection

In addition to the HIV-1 subtypes described in a previous publication (Greve et al., 2009), the primer system was used for the detection of additional subtypes (F1/F2, National Institute for Biological Standards and Control (NIBSC) Nr.: ARP 1034; CRF G/A, NIBSC Nr.: ARP1042; Subtype C, NICBC Nr.: ARP149 and ARP197; Subtype H, NIBSC Nr.: ARP 175).

2.2. Oligonucleotide design

The primer design was published elsewhere (Cleland et al., 2001; Drosten et al., 2006; Holmes et al., 2001; Rouet et al., 2007; Somogiy, 2002) and generates a fragment with the sequence of two highly conserved regions in the 5′ LTR region of the HIV-1 genome. These primers allow only for specific amplification of a single target fragment. The test described herein is based on a three primer system (Greve et al., 2009) and in this way it enables the amplification of both an internal control sequence and the corresponding provirus sequences.

2.3. Internal control (DNA HIV-1 standard)

The same internal control was used as described by Greve et al. (2009) except the last translation step into RNA. The DNA internal control was used for the extraction control of the DNA and for the PCR

2.4. HIV-1 DNA enrichment and isolation

An aliquot of 200–500 μl of the blood sample was diluted in 2 ml PBS/BSA and overlaid with 3 ml lymphocyte separation medium (lympho separation medium, MP Biomedicals, OH, USA). Cells were separated by centrifugation at 400 × *g* for 20 min. The separated lymphocytes were carefully collected and washed by adding 12 ml PBS and centrifuged again at 400 × *g* for 10 min.

Finally, the pellet was dissolved in freshly prepared lysis-buffer (QiAmp DNA Mini Kit, Qiagen, Germany) and treated as recommended by the manufacturer.

2.5. Amplification

In order to avoid inadvertent carryover of fragments amplified previously, dUTPs instead of dATPs were used in any PCR amplification and uracil-DNA glycosylase (UDG) to digest possible cross contaminants (Pang et al., 1992). The reaction was carried out at 37 °C for 15 min before PCR initiation. The PCR master mix included the following components in each 25 μl reaction: 2.5 μl 10× buffer; 0.5 μl of the dNTP mix including 5 mM dUTPs, 5 mM dTTPs and 10 mM dATPs, dGTPs, dCTPs, 0.5 μl UDG 1 U/μl (Bioron, Ludwigshafen, Germany); 0.1 μl SuperTaq 5 U/μl (HT Biotechology, UK); 0.1 μl Taq Antibody 1.1 μg/μl (Clontech, Heidelberg, Germany); 5 pmol target specific DIG labelled forward primer; 5 pmol internal control specific DNP labelled forward primer and 5 pmol BIO labelled reverse primer (Eurogentec, Seraing, Belgium). For the detection of provirus DNA 100–600 ng (Vitone et al., 2005) of the isolated DNA and 100 copies standard DNA was used which was taken through the entire sample preparation procedure. Amplification of the target fragment was carried out in PCR reaction tubes in an Eppendorf mastercycler using the following protocol: the specificity of the reaction was increased using the "Touch Down" (TD) PCR (Don et al., 1991; Korbie and Mattick, 2008; Zumarraga et al., 2005). The annealing temperature is six degrees above the calculated temperature of the primer pair and decreased over six cycles by one degree/cycle. After denaturation at 95 °C for 10 min and inactivation of the UDG, TD took place for six cycles from 64 °C down to 58 °C. Additional 39 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and extension at 72 °C for 4 min were used for yes/no answer of the provirus detection. For the quantification of PCR-products only 33 cycles were used after initial six TD cycles. This ensures that the PCR amplification resides in the exponential phase.

2.6. PCR stability

To control the stability of the provirus PCR, the fragment of interest was amplified out of 200, 400, 600, 800, and 1000 ng of isolated human genomic DNA from a HIV-1 infected patient. The same amount of DNA was also amplified from a non infected person to ensure that no nonspecific fragments or satellite bands without the provirus DNA were generated. The amplification was controlled by gelelectrophoresis (CyFox, Partec, Görlitz, Germany).

2.7. PCR sensitivity

To verify the sensitivity of the PCR system, 1000, 500, 100, 50, and 10 copies of the internal standard were amplified and controlled by gel-electrophoresis. A standard curve was generated by flow cytometric quantitation of the above listed standard. Each copy number was amplified and measured five times. The standard curve was used to determine proviral load of 14 proviral DNA samples of persons infected with HIV.

2.8. Purification of the PCR product

As recommended by the manufacturer, 20 μl of the PCR product was purified with the omnipure-OLS System (OMN Life Science, Bremen, Germany) to remove unused primers.

2.9. Beads binding and flow cytometry

The beads binding procedure and the flow cytometric measurement were performed as described previously (Greve et al., 2009).

2.10. Statistical analysis

A standard curve was generated by five independent amplifications and subsequent flow cytometric measurements of the DNA standard. Regression analysis and calculation of the formula was done by using the Origin 7.5G Software (OriginLab Cooperation, Northhampton, USA). FloMax (Quantum Analysis, Münster, Germany) was used to acquire and process flow cytometric data.

3. Results

3.1. Test specificity

In addition, subtype C (Zambia and Tanzania) subtype H (Zaire), CRF A/G and F1/F2 were evaluated (Fig. 1). The internal DNA

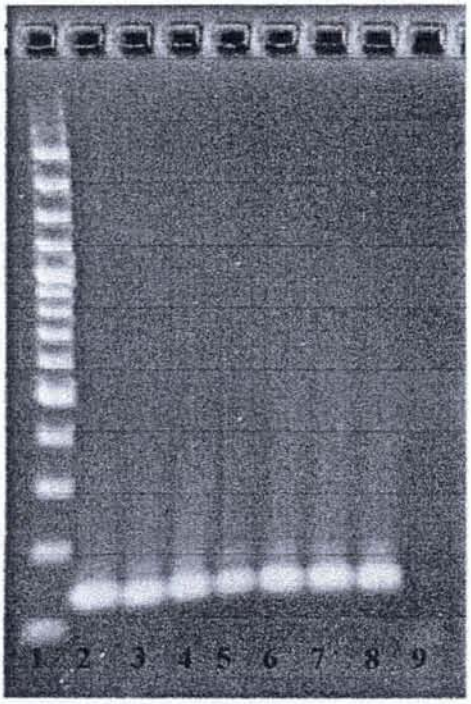

Fig. 1. Five NIBSC HIV-1 subtypes were amplified to verify the specificity of primer system used. Lane 1: 100 bp ladder; lanes 2 and 3: pos. control; lane 4: subtype A/G; lane 5: subtype F1/F2; lane 6: subtype C, Zambia; lane 7: subtype C, Tanzania; lane 8: subtype H, Zaire; lane 9: neg. control.

standard with HIV-1 5′LTR specific sequences ensured the correct course of purification and amplification.

3.2. PCR stability

TD was used to detect proviral DNA of HIV-1 out of 200–1000 ng of human genomic DNA from infected patients. Regardless of the large amount of human DNA, no amplification reduction of the specific provirus target fragment was visible (**Fig. 2**). Samples from individuals negative for HIV were subjected to the same amplification conditions to ascertain that no nonspecific fragments or satellite bands were generated (**Fig. 2**).

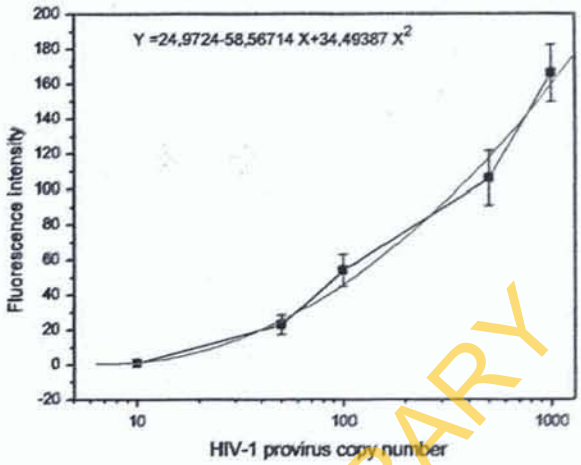

Fig. 3. PCR amplification of known copy numbers of the DNA standard (10 to 10^3 copies) and quantification by flow cytometry. Standard curve and regression analysis (red line and formula) are given. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.3. PCR sensitivity

To verify the lower limit of detection, 1000, 500, 100, 50, 10, copies of the internal standard were amplified and the PCR was stopped 33, 34 and 35 cycles after six initial TD-cycles. The amplification products were visualized by gelelectrophoresis (picture not shown). Quantitation took place on a flow cytometer after 39 PCR cycles (including the six TD cycles) (**Fig. 3**).

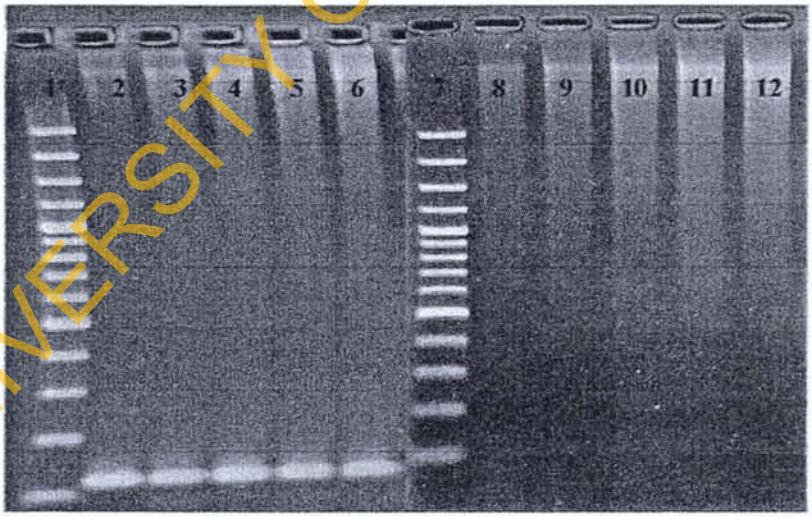

Fig. 2. Provirus amplification out of 200–1000 ng human DNA of a HIV-1 infected patient. Lane 1: 100 bp ladder; lane 2: 200 ng DNA; lane 3: 400 ng DNA; lane 4: 600 ng DNA; lane 5: 800 ng DNA; lane 6: 1000 ng DNA. High amounts of human DNA are not inhibiting the amplification of the proviral PCR fragment. The same PCR conditions were used for the DNA amplification of an uninfected person. No cross reaction or satellite bands are detectable. Lane 7: 100 bp ladder; lane 8: 200 ng DNA; lane 9: 400 ng DNA; lane 10: 600 ng DNA; lane 11: 800 ng DNA; lane 12: 1000 ng DNA.

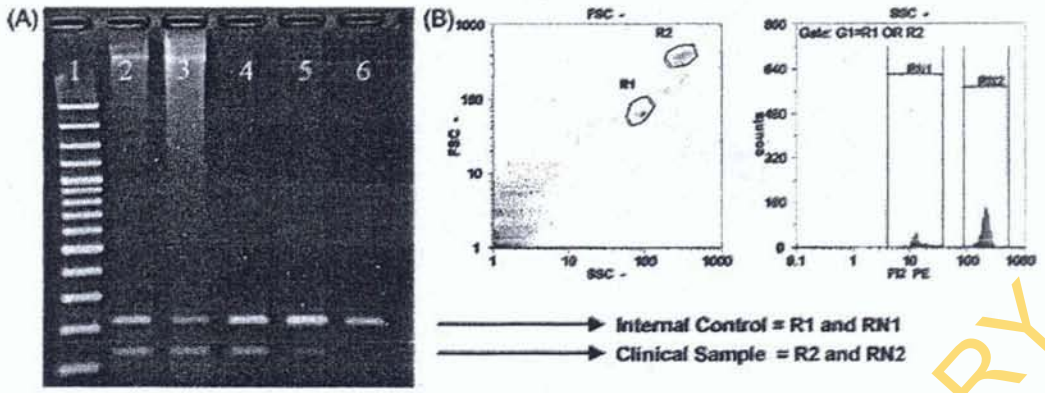

Fig. 4. (A) Co-amplification of the internal control and proviral DNA. Lane 1: 100 bp ladder; lanes 2 and 3: example of two HIV-1 proviral DNA from Germany; lanes 4 and 5: example of two HIV-1 proviral amplifications from Africa; lane 6: negative control. (B) Example of the detection of HIV-1 proviral DNA and the internal control by flow cytometry. R1 and RN1 indicates the internal control and R2 and RN2 the PCR-product of the positive clinical sample.

3.4. Detection by gelelectrophoresis and flow cytometry

The fragments of the internal control and the clinical samples were separated by electrophoresis on a 2% agarose gel, stained with ethidiumbromide and visualized with a UV transilluminator (Fig. 4A).

The flow cytometric measuring principle is shown by the analysis of a positive HIV-1 sample with the detection of the provirus and the internal control sequence (Fig. 4B).

The above methods were used to test blood samples of 60 persons infected with HIV from Ibadan Nigeria, 20 positive samples from Germany ($n = 80$) and 20 non-infected individuals for their proviral DNA. Gelelectrophoresis, and flow cytometry showed the same yes/no results, indicating that depending on the equipment available in a laboratory both methods could be used. All the control samples of uninfected individuals were negative for HIV-1 proviral DNA indicating 100% specificity of the assay. HIV-1 proviral DNA of 14 samples (each 500 ng genomic DNA) was amplified and quantified by flow cytometry (Table 1).

4. Discussion

The persistence of proviral human immunodeficiency virus Type 1 reservoir represents one of the major drawbacks to the total eradiation of HIV-1. The reason of persistence of HIV infection in the face of current therapy appears to be multifactorial and latent but replication-competent provirus are resting in CD4$^+$ T cells and other CD4 positive leucocytes. Clearance of HIV infection will almost certainly require many different approaches that includes potent suppression of HIV replication, therapies that reach all compartments of residual HIV replication and depletion of any reservoirs of persistent. In addition, reliable monitoring of all important parameters of HIV infection such as CD4$^+$ T-cell counts, CD4$^+$ %, viral load, and proviral DNA quantitation is necessary. Therefore, the detection of proviral HIV-1 DNA is one important diagnostic marker especially when the HIV-1 RNA levels fall below the detectable limits of assays during HAART. Furthermore, the detection of HIV-1 proviral DNA is an important diagnostic marker for the evaluation of HIV-1 infection of newborns of women seropositive for HIV-1 as well as detection of HIV during the pre seroconversion window period. Similar to the problems with viral load measurement in serum samples, there is currently no commercial test for detection of all known HIV-1 subtypes or circulating recombinant forms. Some "in house" tests have been published (Drosten et al., 2005; Gibellini et al., 2004) but in routine use, the overall costs are extremely high and the required laboratory equipment is very complex and expensive. Therefore, the published viral load test was optimized (Greve et al., 2009) to amplify proviral DNA from all known HIV subtypes and CRFs. The system was established and its specificity, sensitivity and reliability evaluated. The specificity was determined by amplifying the same subtypes from the NIBSC as reported previously by Greve et al. (2009).

Table 1
Quantification of provirus DNA in 14 proviral positive samples by flow cytometry. All samples were detected positive by gel-electrophoresis and real-time PCR (SYBR Green).

Sample	FC (copies per 500 ng genomic DNA)	GE	RT
1	103 copies	Positive	Positive
2	44 copies	Positive	Positive
3	47 copies	Positive	Positive
4	45 copies	Positive	Positive
5	155 copies	Positive	Positive
6	130 copies	Positive	Positive
7	55 copies	Positive	Positive
8	195 copies	Positive	Positive
9	179 copies	Positive	Positive
10	185 copies	Positive	Positive
11	290 copies	Positive	Positive
12	150 copies	Positive	Positive
13	149 copies	Positive	Positive
14	67 copies	Positive	Positive

FC, flow cytometry; GE, gel-electrophoresis; RT, real time PCR (SYBR Green).

The use of a "Touch-Down" (TD) PCR described above helped to overcome the problem of primer mismatch and the production on non-specific PCR-products which become obvious in the presence of high amount of human DNA. By this modification, false positive results are prevented.

Four of the 80 samples from the infected cohort that showed no amplification were identified to have a DNA content (<8 ng/μl). It should be noted that 4–6 μl of the isolated DNA per PCR was used. Gueudin et al. (2008) had shown that provirus DNA load higher than 3.5 log10/μg DNA (3162 copies/μg DNA) are rarely events in persons infected with HIV. This means that the DNA proviral load maximum is about three to four copies per ng DNA. In case of a proviral load of <100 copies per μg DNA the detection limit is about 40–60 ng DNA which may partly explain the reason for non-amplification of the target fragment in these four samples.

5. Conclusion

A PCR-based test to amplify proviral DNA in samples of persons infected with HIV with high sensitivity and specificity was

developed. All known HIV-1 subtype sequences and CRFs become amplified; the test is reliable under high amounts of human DNA and is workable by using standard gel electrophoresis, flow cytometry or real-time PCR (SYBR Green).

In addition to the "yes" and "no" detection of proviral DNA it is possible to quantify the proviral load by flow cytometry. Laboratories equipped with a flow cytometer, normally used for $CD4^+$ and $CD4^+$ % could use the same device for proviral load.

On the basis of the method herein described, a PCR-based test for the determination of the viral load of HIV-2 and provirus DNA is in progress. The goal is to measure all relevant parameters for the assessment of HAART, to reduce the equipment requirement and the costs per test substantially.

Conflict of interest

The authors declare no conflict of interest or financial relationship influencing the conclusions of the work.

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