Fluorometric detection of HIV-1 genome through use of an internal control, inosine-substituted primers, and microtiter plate format

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We describe a PCR-based fluorometric assay for the detection of the HIV-1 genome. This technique consists of a reverse hybridization with oligonucleotide probes covalently coated onto a microtiter plate as a solid support. Several improvements to the PCR amplification and detection steps gave greater sensitivity and specificity for HIV-1 screening and resulted in a convenient and rapid technique. False-positive results were avoided by using uracil DNA glycosylase. False-negative results from the presence of PCR inhibitors were detected by coamplifying an internal control with the viral sequence. False-negative results from viral genome variability were limited by using two pairs of primers and by incorporating inosine at the primer positions corresponding to viral polymorphic nucleotides. Furthermore, the hybridization buffer and enzymatic reaction were optimized to increase the assay's sensitivity. The sensitivity and specificity of the fluorometric detection were similar to those of radioisotopic oligonucleotide solution hybridization; however, hands-on time was reduced, and the use of radioactivity was eliminated. We have used this technique routinely on 115 samples and obtained 100% specificity and high sensitivity (only one false-negative result) according to viral culture and (or) serological status of the patients.

INDEXING TERMS: human immunodeficiency virus • reverse hybridization • screening, neonatal • polymerase chain reaction • biotin-streptavidin interaction

The polymerase chain reaction (PCR) has proved useful for the follow-up of HIV-1 infection [1–7] and especially for neonatal diagnosis of children born to HIV-1-infected mothers [8–11]. HIV-1 infection is usually diagnosed serologically by the presence of specific antibodies or p24 HIV-1 antigen. However, serological methods are not suitable for detecting HIV-1-infected infants born to HIV-1-infected mothers because the presence of maternal antibodies during the first months of infancy hampers serological diagnosis. Coculture techniques, which detect the presence or absence of the virus [11], are independent of the serological status of the patient but do not produce useful results for several weeks. A more rapid technique is needed for quick detection and better follow-up of infected neonates. Because PCR screens directly for the presence of proviral DNA or viral RNA, it is suitable for HIV detection and quantification in newborns born to HIV-1-infected mothers. Moreover, this technique is extremely sensitive and yields results within a few hours.

PCR amplification is very specific and sensitive, but its application to the diagnosis of HIV-1 infection raises some difficulties. First of all, the copy number of HIV-1 proviral DNA can be very low, so the sensitivity of the PCR must be optimized. Moreover, the poor replication fidelity of HIV-1 is such that variability occurs even in the viral DNA coding sequences; mispriming because of these viral DNA mutations could lead to false-negative results. Further, when negative results are obtained, the presence of PCR inhibitors must be excluded.

The first methods described for the detection of PCR-amplified HIV-1 DNA were based on radioisotopic oligonucle-
otide solution hybridization (OSH) [11, 12]. This method has high specificity and sensitivity [11, 13] but lacks the convenience of a nonradioisotopic test and cannot be automated because an electrophoresis step is used. Several convenient commercial kits using nonradioactive detection methods have been developed, either in a microtiter plate format (Roche Diagnostic Systems or Du Pont) or in polypropylene tubes (Gen-Probe). Both formats are compatible with automation for large sample throughput and show good sensitivity and specificity; however, they do not take into consideration the possibility of viral genome variability or the presence of a Taq polymerase inhibitor in preparing the DNA sample.

In this study, we combined several technical improvements to optimize the sensitivity and specificity of the HIV PCR amplification. We also developed a fluorometric detection method for PCR-amplified proviral DNA in a microtiter plate format. Taq polymerase inhibitors were detected by including an internal control that is coamplified with the viral sequence. False-negative results from viral genome variability were reduced by using two primer pairs substituted with inosine at polymorphic nucleotide positions of the viral genome. Because inosine is able to hybridize to all nucleotides, it increases the annealing of primers with targets that could have mismatches at the positions where inosine has replaced the original bases [14, 15]. PCR contamination yielding false-positive results was avoided by the use of uracyl DNA glycosylase (UDG) [16]. Biotinylated PCR amplification products were hybridized to oligonucleotide probes that were covalently coated onto a microtiter well. Hybrids were detected with a streptavidin–alkaline phosphatase conjugate, which generates a fluorescent product.

We first compared the sensitivity and specificity of this test with those of the OSH method. We then performed this test routinely for 6 months and compared the results with the results obtained by viral culture or with the serological status of the patients.

Materials and Methods

BLOOD SAMPLE PREPARATION

Clinical samples were obtained from children born to HIV-1-infected mothers and from their mothers. Peripheral blood mononuclear cells (PBMC) were obtained from whole blood collected into EDTA-coated tubes. The samples were prepared in a room where no HIV-1 PCR product had been handled. The PBMC were separated by centrifugation on a Ficoll gradient and washed three times in a 9 g/L NaCl solution. The pellet was washed twice in 1 mL of an erythrocyte lysis buffer: 10 mmol/L Tris-HCl, pH 7.5, containing 0.32 mol/L saccharose, 5 mmol/L MgCl2, and 10 g/L Triton-100 (Sigma, St. Louis, MO). The suspension was centrifuged at 7000g for 5 min. The cells were resuspended in 9 g/L NaCl solution and counted. After centrifugation again, the pellet was resuspended in leukocyte lysis buffer—50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 7.5), 2.5 mmol/L MgCl2, 4.5 g/L Nonidet P40 (Sigma), and 4.5 g/L Tween 20 (DIFCO, Detroit, MI)—to a final concentration of 107 cells per milliliter of buffer. This suspension was incubated at 55°C for 1 h with proteinase K (Boehringer Mannheim, Mannheim, Germany), 100 mg/L/ L. The enzyme was then inactivated at 100°C for 10 min, and samples were stored at −20°C.

To control for the efficiency of the DNA extraction, we performed PCR with genomic primers on all samples. Briefly, a HLA-DQA1 gene amplification was performed on each sample. The reaction contained 50 pmol of each primer (HLA-DQA1: GGT GTA AAC TTG TAG CAG, and HLA-DQA2: GGT AGC AGC GGT AGA GTT G), 4 mmol/L MgCl2, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.5), and 200 μmol/L each of dATP, dCTP, dGTP, and dTTP. One unit of Taq polymerase (Perkin-Elmer, Foster City, CA) was added when the mixture was at 80°C. After an initial denaturation of 3 min, 30 cycles were performed, each being as follows: 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C. A band of 222 bp was visualized on a 1.5% agarose gel by staining with ethidium bromide.

ASSAY COMPONENTS

Primer selection. The two pairs of primer used, SK38i/SK39i and GAG04/GAG06 (Table I), were both located in the HIV-1 GAG gene. The SK38i/SK39i primer pair is derived from the previously described SK38/SK39 primer pair [11, 17]. The HIV-1 polymorphic nucleotide positions in the primers were found by performing a sequence alignment of 15 strains of HIV-1 by using the Clustal software (Fig. 1). They were obtained from GenBank (genetic sequence databank, National Center for Biotechnology Information, Bethesda, MD) and consisted of HIVBRUCG, HIVEILCG, HIVH3CG, HIVJRCSF, HIVMALCG, REHTLV3, AIARV2, HIV3BH5, HIVOY, HIVRF, REHIHVXB, REHIVJH3, REHIVMNC, REHIVNLA, and REHVIZZ2. We incorporated 3 inosines into primer SK38i and 4 inosines into primer SK39i, corresponding to, respectively, positions 3, 18, and 24 and positions 3, 6, 9, and 23 from the 5′ end of the primer.

The GAG04 and GAG06 inosine-substituted primers were as described by Piatak et al. [7, 14]. Primers SK38i and GAG04 were biotinylated at the 5′ end during oligonucleotide synthesis.

SK38i/SK39i internal control preparation. The internal control was obtained by PCR by using a mutational primer that deleted 14 bp in the sequence of the native HIV-1 GAG gene (Fig. 2A). The SK39i primer is located at the 3′ end of the amplified region. The mutational primer is located at the 5′ end of the amplified region, at position +1132 to +1148 of the GAG gene. At the 5′ extremity, the mutational primer has a tail composed of the SK38i primer sequence that is complementary to the region +1090 to +1116 of the GAG gene (Table I). Thus, amplification of such a PCR product with SK38i/SK39i yields a PCR product 14 bp shorter than a native HIV-1 PCR product.

The PCR was performed by using a previous SK38i/SK39i PCR product. The reaction contained 20 pmol of SK39i and 20 pmol of the mutational primer in a volume of 50 μL containing

5 Nonstandard abbreviations: OSH, oligonucleotide solution hybridization; UDG, uracyl DNA glycosylase; PBMC, peripheral blood mononuclear cells; SSC, saline sodium citrate; PEG, polyethylene glycol; 4-MUP, 4-methylumbelliferone phosphate; and pNPP, p-nitrophenol phosphate.
2 mmol/L MgCl₂, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.5), and 200 μmol/L each of dATP, dCTP, dGTP, and dTTP. One unit of Taq polymerase was added when the mixture was at 80 °C. After an initial denaturation of 3 min, 30 amplification cycles were performed, each as follows: 30 s at 95 °C, 30 s at 45 °C, and 30 s at 72 °C. This control was cloned into a PGEM4Z plasmid (Promega, Madison, WI). Plasmids containing the internal control were spectrophotometrically quantified at 260 nm. A solution containing 2 copies of plasmid per microliter was prepared by diluting the plasmids in distilled water; the solution was then stored at −20 °C.

Probes selection. The SK102 probe previously described [12] hybridizes to the GAG04/GAG06 biotinylated PCR products. The viral probe (VP38i/39i) and the internal control probe (IC38i/39i) were chosen so as to specifically detect either the viral or the internal control SK38i/SK39i PCR product (see Fig. 2B). The VP38i/39i and the IC38i/39i probes were designed to specifically hybridize to the PCR products from the viral DNA and the internal control, respectively (Table 1). Cross-hybridization was controlled for, and the two probes were found to be highly specific. We did not incorporate inosine into these probes because the hybridization and washing conditions used here were at low stringency and did not differentiate point mutations.

![Diagram](image)

**Table 1. Summary of oligonucleotide sequences.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Nucleotide sequence</th>
<th>Nucleotide position in HIV-1 BRU strain</th>
<th>Nucleotide position</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK38i primer*</td>
<td>+ 1090–1116</td>
<td>5'-AAI CCA CCT ATC CCA GGA GAI AT-3'</td>
<td></td>
</tr>
<tr>
<td>SK39i primer</td>
<td>+ 1175–1203</td>
<td>5'-TTI GGI CTT GTC ATG GCC AIA ATG C-3'</td>
<td></td>
</tr>
<tr>
<td>VP38i/39i probe</td>
<td>+ 1118–1139</td>
<td>5'-CAG GAT TAT CCA TCT TTT ATA-3'</td>
<td></td>
</tr>
<tr>
<td>IC38i/39i probe</td>
<td>+ 1110–1116 and + 1132–1141</td>
<td>5'-CCC AGG ATT TTC TC-3'</td>
<td></td>
</tr>
<tr>
<td>Mutational primer</td>
<td>+ 1090–1116 and + 1132–1148</td>
<td>5'-AAT CCA CCT ATC CCA GTG GGA ATT AAT CTT GGG ATT AAA-3'</td>
<td></td>
</tr>
<tr>
<td>GAG06 primer</td>
<td>+ 827–854</td>
<td>5'-GCI TTI AGC CCI GAA GTI ATA CCC ATG-3'</td>
<td></td>
</tr>
<tr>
<td>GAG06 primer*</td>
<td>+ 1052–1080</td>
<td>5'-CAT ICT ATT TGT TCI TGG AGG GTA CTA G-3'</td>
<td></td>
</tr>
<tr>
<td>SK102 probe</td>
<td>+ 1001–1034</td>
<td>5'-GAG ACC ATC AAT GAG GAA GCT GCA GAA TGG GAT-3'</td>
<td></td>
</tr>
</tbody>
</table>

*5' biotinylated.

![Diagram](image)

Fig. 1. Sequence alignment of a fragment of the GAG gene in 15 HIV-1 strains. Nucleotide positions correspond to those of HIV-1 BRU strain. *, variable position.

Fig. 2. (A) Preparation of internal control; (B) amplification and detection of the viral genome and internal control.
PCR CONDITIONS FOR HIV-1 AMPLIFICATION

Each sample was subjected to two amplifications performed simultaneously under identical PCR conditions, one with the primers SK38i/SK39i and the other with GAG04/GAG06. PCR reagents were mixed in a laminar flow hood to prevent PCR contamination of the samples. The PCR reaction final volume was 50 µL, and the reagent mixture consisted of 2.5 mmol/L MgCl₂, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.5), 50 pmol of each primer (SK38i + SK39i) or (GAG04 + GAG06), 100 µmol/L dATP, 100 µmol/L dGTP, 100 µmol/L dCTP, 200 µmol/L dUTP, 1 U of Taq DNA polymerase, and 0.5 U of UDG (Gibco BRL Life Technologies, Cergy-Pontoise, France). To each PCR tube, 10 µL of the PBMC lysate containing 10⁷ cells was added with a positive-displacement pipette. For the SK38i/SK39i PCR, 20 copies of the internal standard (in 10 µL) were also added. A blank PCR was run under the same conditions.

Biotin was also incorporated during PCR via dATP-biotin 11 (Gibco BRL) by the Taq polymerase. PCR conditions were the same except that the biotinylated primers SK38i and GAG04 were replaced by unmodified primers, and dATP-biotin 11 (12.5 mmol/L) was added to the PCR mix.

The samples were incubated at 50 °C for 10 min to allow UDG to digest any PCR contamination. The PCR consisted of a 5-min denaturation at 95 °C, followed by 40 cycles, each for 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C. The PCR products were kept at 72 °C, then moved into a separate room for analysis. Without delay, 5 µL of 1 mol/L NaOH was then added to inactivate UDG and to denature the amplified products.

MICROTITER PLATE HYBRIDIZATION ASSAY

The VP38i/39i, SK102 [12], and IC38i/39i oligonucleotide probes were covalently bound to clear microtiter plates [18]. [Coated microtiter plates are commercially available from Genset (Paris, France).] To each well, containing 100 µL of hybridization solution [5 × saline sodium citrate (SSC; 1 x = 0.15 mol/L NaCl, 0.015 mol/L sodium citrate, pH 7.0) + 50 g/L polyethylene glycol (PEG), molecular mass 8000 kDa] preheated at 42 °C, was added 10 µL of denatured PCR product: GAG04/GAG06 PCR product was added to the SK102 probe-coated wells, and SK38i/SK39i PCR product was added to the VP38i/39i- and IC38i/39i-coated wells. For all probes, one well received no PCR product and was used as the negative detection hybridization (= background); another well was used for the PCR negative control.

After 60 min of hybridization at 42 °C, the wells were washed three times with 200 µL (per well) of wash buffer I (2 × SSC + 1 g/L sodium dodecyl sulfate) preheated at 45 °C. A fourth wash was performed at 42 °C for 20 min. The wells were then incubated for 15 min at room temperature with 100 µL/well of the conjugate solution (streptavidin–alkaline phosphatase, available from Genset) diluted 10 000-fold in wash buffer II [0.1 mol/L Tris-HCl, pH 7.5, 0.15 mol/L NaCl, and 10 g/L bovine serum albumin (Sigma)], followed by four washes with 200 µL/well of wash buffer II. Finally, 100 µL of a fluorescent substrate solution [4-[(3-aminopropyl)aminomethyl]benzene-1,3-disulfonate, 10 mmol/L MgCl₂] was added to each well, and the plate was incubated for 15 min at room temperature. Fluorescence was measured at an excitation wavelength of 365 nm and an emission wavelength of 450 nm with a Microfluor fluorometer (Dynatech, Guyancourt, France).

p-Nitrophenyl phosphate (pNPP; Gibco BRL), a colorimetric substrate, was also used under the same conditions but at 4 g/L. Product absorbance at 405 nm was measured with a Dynatech MR500 colorimeter.

Hybridization was also performed in a Denhardt buffer: 5 × SSC containing 5 g/L sodium dodecyl sulfate, 1 g/L Ficoll (Type 400; Pharmacia, Uppsala, Sweden), 1 g/L polyvinylpyrrolidone, and 1 g/L bovine serum albumin.

DATA ANALYSIS

Before beginning interpretation, the signal of the negative detection control (or background) had to be low, <50 arbitrary fluorescence units with our Microfluor fluorometer. Moreover, the negative detection control and the negative PCR control had to have similar values if we were to eliminate false-positive results produced by PCR contamination. Furthermore, the signal obtained with the internal control probe (IC38i/39i) had to be at least 5 times that of the negative detection control to eliminate the possibility that a PCR inhibitor was present in the tested sample.

Because the value of the negative detection control could vary with the conservation state of the substrate (possibility of spontaneous dephosphorylation), we interpreted each experiment according to its background value. A sample was considered negative when the signals from both viral probes VP38i/39i and SK102 were less than twice that of the negative detection control. The negative cutoff value was determined by studying a population of 100 HIV-1-negative controls. Results from each experiment were expressed as the ratio of fluorescence emission, sample/background. For example, in a series of 25 HIV-1-negative controls, the mean (± SD) ratio for sample/background was 1.16 ± 0.19 and thus less than two times the background. In HIV-1-negative controls, the mean value ± 3 SD was always less than twice the negative control background.

A sample was considered positive if one of the two viral probes had a signal ≥5 times that of the PCR negative control. The positive cutoff value we used was determined by studying 40 HIV-1-infected patients. The lowest values were always >5 times the background. When the fluorescence emission of a sample ranged between 2 and 5 times the background, we performed a second PCR to confirm or rule out infection with HIV-1.

OLIGONUCLEOTIDE SOLUTION HYBRIDIZATION

OSH was performed as previously described [11, 12]. PCR products were precipitated with ethanol. After centrifugation, the pellets were dissolved in 10 µL of "FABH" buffer: 800 mEq/L deionized formamide, 0.4 mol/L NaCl, 40 mmol/L 1,4-piperazinediethanesulfonic acid (pH 6.4), and 1 mmol/L EDTA (pH 8.0). The SK19 oligonucleotide was 5'-end labeled with [γ-32P]ATP by use of a polynucleotide kinase. We added 5 ng of 32P-end-labeled SK19 probe to the reaction mixture,
covered the mixture with oil, denatured the reactants at 100 °C for 5 min, and then let the hybridization proceed at 50 °C for 1 h. After electrophoresis of the reaction products on a 10% polyacrylamide gel, we detected hybridization by autoradiography on Kodak XAR-5 film for 12 h.

**Results**

**TECHNICAL CHARACTERISTICS**

Taq polymerase inhibitors such as hemoglobin, high DNA concentration, or cellular membranes can lead to false-negative results. These inhibitors were detected by the internal control that was added to the PCR SK38i/SK39i reaction. Two copies of the internal control could be detected with this technique. However, when the internal control was amplified with the 10^3 PBMC cell lysate, 20 copies were necessary to obtain reproducible results. This quantity was kept low to favor viral amplification and to be sensitive to any PCR inhibitor. Because the internal control always had to be positive, we could thus distinguish negative samples from nonamplified samples.

Using a biotinylated primer instead of dATP-biotin incorporation during PCR increased the sensitivity of the assay (Table 2). The optimized ratio of dATP-biotin/dATP for the incorporation of dATP-biotin by the Taq polymerase theoretically would average 2.8 biotin molecules per target molecule, in contrast to 1 biotin molecule with the use of biotinylated primers; however, using biotinylated primers improved the positive signals 11-fold. Apparently, Taq polymerase does not incorporate dATP-biotin and dATP with the same efficiency. Sensitivity was also increased by optimizing the conditions of hybridization with the probe. Table 2 also shows the influence of the hybridization buffer on the intensity of the positive signals. The two hybridizations were performed in parallel with the same PCR products and hybridization conditions except for the hybridization buffer. The hybridization rate with the Denhardt buffer is only one-third of that with the PEG buffer. Both buffers have the same sodium concentration.

Finally, we compared a fluorescent substrate, 4-MUP, and a colorimetric substrate, pNPP, to improve the sensitivity and (or) the rapidity of our test. Table 2 shows the positive control/negative control ratio of results obtained with the two substrates at different enzymatic reaction times. The 4-MUP fluorescent substrate seemed to be more convenient than the colorimetric substrate and gave a fivefold higher controls ratio for a 15-min reaction time (11.5 vs 2.5). Chemiluminescent substrates, such as dioxetane substrates, were not tested because they need a specific detection system less widely used in clinical laboratories.

**FLUOROMETRIC DETECTION AND RADIOISOTOPIC OSH COMPARISON**

The sensitivity of the fluorometric detection system was first compared with that of radioisotopic OSH by retrospectively studying 38 OSH-positive samples. Using the SK38i/SK39i primers, we performed a PCR amplification of cell lysates from these samples and detected the fluorescence of the biotinylated PCR products after hybridization to the SK38i/SK39i probe. All 38 samples were positive by the fluorometric detection system.

Moreover, a prospective study of 70 samples by both methods gave 100% correlation between the OSH method and the fluorometric test. Among the 70 samples, 18 were positives, and the signal intensities were comparable for both methods (Fig. 3).

**CLINICAL TRIAL: A PROSPECTIVE STUDY**

We have been routinely using fluorometric detection for 6 months and have tested 115 samples. Table 3 shows some typical results obtained with our technique, and all the results obtained with the SK38i/SK39i primers, expressed as sample/background ratio, are plotted in Fig. 4 (results obtained with the GAGO4/ GAGO6 primers were very similar). The samples were from 10 known infected mothers and 54 newborns born to HIV-1-infected mothers, and some patients were followed for several

| Table 2. Sensitivity optimization of the microtiter plate hybridization and detection assay for method of biotinylation, hybridization buffer, and fluorescent (4-MUP) vs colorimetric substrate (pNPP). |
|---|---|---|
| **Fluorescence units** | Pos./neg. ratio | Color. Fluor. |
| dATP-biotin incorporation | 290 | 100 |
| Biotinylated primer | 390 | 120 |
| Denhardt buffer | 670 | 160 |
| PEG buffer | 1800 | 200 |
| Enzyme reaction, minutes | | |
| 15 | 2.5 | 11.5 |
| 30 | 3.0 | 17.3 |
| 40 | 5.3 | 20.6 |
| 70 | 8.6 | 27.1 |
| 120 | 11.8 | 28 |
| 150 | 12.3 | 26.7 |

*5× Denhardt + 5× SSC + 5 g/L sodium dodecyl sulfate.
*5× SSC, 50 g/L PEG.

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**A.**

**B.**

![Fig. 3. Comparison of the fluorescent microtiter plate assay (B; fluorescence units) with the OSH technique (A): lanes 1 to 5, patients 1 to 5; lane 6, blank PCR; lane 7, positive control; lane 8 (panel B), blank well.](image-url)
months with this technique. The samples were amplified with the SK38i/SK39i and the GAG04/GAG06 primer pairs and were also tested by viral culture and (or) HIV-1 serology [4].

Among the 115 samples tested, 18 were positive; of these, 16 were positive with both primer pairs, and 2 were positive with the GAG04/GAG06 primers but negative with the SK38i/SK39i primers. The PCR results agreed well with the results of viral culture, HIV-1 antibody detection, or p24 antigen detection (P <0.01, Kappa test). In only two cases were discrepancies found. PCR was positive for one newborn for whom the simultaneous viral culture and p24 antigen detection were negative; however, HIV-1 infection of this patient was confirmed by a positive viral culture at age 3 months. The one false-negative sample was from a seropositive mother for whom the amplification with the two pairs of primers was negative despite a positive signal for the internal control. Follow-up of the 54 children for 6 months did not reveal any false-negative or false-positive initial PCR results.

The sensitivity of this fluorometric microtiter detection assay was 99% (one false-negative result among 115 tested samples) and specificity was 100% for this prospective study.

**Discussion**

Several PCR-based methods for detecting HIV-1 DNA without the use of radioisotopes have been developed by Roche Diagnostic Systems, Du Pont, and Gen-Probe, but these commercial kits lack the controls and safeguards that we have incorporated into this test. The UDG system, an effective safeguard against contamination by previous PCR products [7], is only proposed in the Roche detection system. Because of the high variability of the HIV-1 viral genome, the amplification of two different regions theoretically improves the sensitivity of the detection. However, only the Gen-Probe kit supplies two independent primer pairs. Finally, none of these kits uses primers containing inosine at polymorphic nucleotide positions or includes an internal control to detect false negatives that result when a Taq polymerase inhibitor is present.

We combined several technical improvements to optimize the sensitivity and the specificity of the test. The specificity is ensured by hybridization of the amplified samples to HIV-1-specific probes (SK102 and VP38i/39i). The clear microtiter plate format was chosen because it can easily be automated in an ELISA automation system, with either a colorimetric or a fluorometric substrate. The terminal binding of the oligonucleotide probes ensures optimal hybridization because the nucleic acid bases do not participate in the linkage to the solid phase and because steric hindrance due to the plastic surface is reduced. No prehybridization or special surface treatment is necessary before using the plates, which allows for a simple and rapid detection protocol.

To increase the assay sensitivity, we optimized the hybridization step and the detection of the PCR product. We also took into consideration the high rate of HIV-1 DNA mutation and the possibility of a Taq polymerase inhibitor in cell lysate.

The hybridization yield was improved threefold without increasing the background by eliminating the Denhardt solution from the hybridization buffer and by introducing PEG. The Denhardt-based hybridization buffer is especially intended for membrane hybridization, where nonspecific binding is considerable. In contrast, PEG, which artificially increases the concentration of PCR products during hybridization, could be used with no increase in background because of the low nonspecific binding in microtiter plates. Thus, PEG buffer gave the higher hybridization rate and sensitivity in our assay. The 4-MUP fluorescent substrate was selected because it gave a fivefold higher positive control/negative control ratio at 15 min than did a colorimetric substrate. Finally, use of a biotinylated primer was more sensitive than PCR incorporation of dATP-biotin.

The high variability of the HIV-1 genome could be responsible for false-negative results. To increase the sensitivity of HIV-1 DNA detection, we used two different pairs of primers and replaced the variable nucleotides by inosine [14, 15]. At the known positions of variable nucleotides in the primers we substituted inosine, which is able to hybridize to all nucleotides. However, new mutations could appear and hamper the annealing of the primers. The use of two different pairs of primer theoretically should diminish the sensitivity of the HIV-1 detection method for such variables. In fact, however, we observed in two cases a positive PCR result with the GAG04/ GAG06 primers when the SK38i/SK39i result was negative. Because we did not sequence the HIV-1 GAG gene, we were not
Moreover, methods specifically involving UDG, of contamination, results.

PCR-based way we synthesized also to the same authors coamplified mutations. able to demonstrate whether these results were caused by new mutations.

False-negative results caused by the presence of PCR inhibitors in the cell lysate were detected by the use of an internal control. The internal control and the proviral DNA were coamplified with the same pair of primers. These targets have an equivalent PCR efficiency because the two PCR products have a similar sequence except for a 14-bp deletion in the internal control. Twenty copies of the internal control were added before PCR: Because the HIV-1 burden is low, amplification of the 20 copies will be sensitive to any PCR inhibitors. Some authors have coamplified cellular internal controls (single copy of a human gene or endogenous RNA) with the HIV-1 genome to control for the presence of PCR inhibitors. Those controls also test for the DNA or RNA extraction. Instead, we chose a synthetic control because the viral burden could be very low in comparison with the genome copy number. Moreover, because we used the same primer pairs, the influence of PCR inhibitors would affect amplification of the HIV-1 genome in the same way as that of the internal control. To test for the DNA extraction step, we systematically performed one genomic PCR (an HLA gene amplification) as described in Materials and Methods.

PCR contamination is an important problem in routine PCR-based diagnosis because it can generate false-positive results. PCR contamination is mostly caused by aerosol carryover from previous PCR products. To avoid such PCR contamination, we lysed the cells and performed the PCR reaction in a room where no PCR product had been handled. Moreover, we performed all amplifications with dUTP instead of dTTP and, before each amplification, used the enzyme UDG, which specifically destroys all DNA fragments containing dUTP. Thus, potentially contaminating PCR products were specifically destroyed.

The 100% correlation between the method we present and radioisotopic OSH in both a retrospective and a prospective study, as well as the 100% specificity and 99% sensitivity of our method for screening 115 samples in a clinical trial, affirm the efficacy of this approach.

In summary, this sensitive and specific nonradioactive procedure detects the PCR-amplified HIV-1 proviral genome. All samples are amplified simultaneously in two different PCR mixes with two pairs of primers, SK38i/SK39i and GAG04/GAG06, and an internal control is included in the SK38i/SK39i amplification. Biotinylated PCR product is reverse-hybridized to oligonucleotide probes (VP38i/39i, IC38i/39i, and SK102) covalently attached to microtiter plate wells. The biotin of the hybridized PCR products is recognized by a streptavidin–alkaline phosphatase conjugate, which generates a fluorescent product by dephosphorylating an optically neutral substrate, 4-MUP. This technique is convenient, safe, and rapid, yielding results in 5–6 h. It is particularly adapted to situations in which pathogens need to be detected directly, such as in neonates born to HIV-1-infected mothers, where maternal antibodies interfere with serological detection. This technique has been optimized to HIV-1 detection but can easily be used for other pathogens and genetic targets.

References


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