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Detection of Human Immunodeficiency Virus Type 1 by Using the Polymerase Chain Reaction and a Time-Resolved Fluorescence-Based Hybridization Assay

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The polymerase chain reaction (PCR) has many potential applications in the field of nucleic acid diagnostics. In particular, it has been successfully applied to the detection of pathogens present in low copy numbers such as the human immunodeficiency virus type 1. Here we describe a time-resolved fluorescence-based hybridization assay which, combined with the PCR, offers an extremely sensitive method for the detection of nucleic acids. In this assay format, the PCR is run by standard procedures and the subsequent hybridization reaction is carried out in solution by using two oligonucleotide probes, one biotinylated and one labeled with europium (Eu^{3+}). The sandwich hybrids are then collected onto a streptavidin-coated microtitration well, and the bound Eu^{3+} is measured in a time-resolved fluorometer. This assay is rapid, user friendly, and quantitative and lends itself to automation. The application of this assay to the detection of human immunodeficiency virus type 1 is described.

Because of its high specificity, DNA probe hybridization is a powerful tool for diagnosing a variety of diseases. It has potential application in the areas of infectious diseases, genetic diseases, oncology, forensics, and disease susceptibility testing. However, the detection sensitivity required for most of the applications has been an obstacle to further test development. This obstacle has been circumvented by the use of various recently described nucleic acid amplification schemes (15, 17, 24, 25, 29). The polymerase chain reaction (PCR) is the most widely used method for amplifying a known fragment of nucleic acid and has found many interesting applications in areas other than DNA probe diagnostics (7).

A wide variety of methods for the detection of PCR-amplified DNA fragments has been described previously (1, 2, 5, 6, 13, 26). Methods allowing for quantification of the original amount of nucleic acid present in the sample have also been presented (21, 28). Many of these methods, however, are still cumbersome, including either the use of electrophoresis or radioactive labels, and are thus not easily automated.

The special demands of DNA probe diagnostics have stimulated development in the field of nonradioactive marker technologies, and progress has been rapid in this area in the last decade. The time-resolved (TR) fluorescence methodology utilizes the long-lived fluorescence of lanthanide ions such as europium (Eu^{3+}), samarium (Sm^{3+}), and terbium (Tb^{3+}). This technology has already been successfully applied to a wide range of nonradioactive assays (18), including a commercial line of immunoassay kits of the DELFIA system (Wallac Oy, Turku, Finland). Applications of TR fluorescence in the field of DNA probe technology have recently been described (3, 4, 20). In this article we describe in detail a method for the labeling of oligonucleotides with Eu^{3+} chelates and show the application of these probes to an affinity-based collection (ABC) hybridization methodology. Two oligonucleotide probes, one Eu^{3+} labeled (Eu^{3+}-S9) and the other biotin labeled (bio-S11), are allowed to hybridize simultaneously in solution to adjacent regions on the target DNA fragment, and subsequently the sandwich hybrids are collected onto microtitration wells coated with streptavidin. The bound Eu^{3+} is measured in a TR fluorometer.

The aim of this study was to create a nonradioactive, sensitive, and quantitative method for the detection of specific nucleic acids by combining the PCR technology and the TR fluorescence-based ABC hybridization assay. We have chosen the human immunodeficiency virus type 1 (HIV-1) as our application example, since this pathogen is present in infected individuals at very low levels and thus represents a diagnostic challenge. It is evident from recent publications that the level of infection correlates with the stage of disease of AIDS (11), and thus a rapid assay allowing direct detection and quantification of HIV-1 is needed.

MATERIALS AND METHODS

Cell samples. Lymphocytes from healthy noninfected donors were isolated by Ficoll-Paque (Pharmacia LKB Biotechnology, Piscataway, N.J.) centrifugation and used as background controls as well as carrier cells in cell dilution experiments.

A COS cell line containing a truncated HIV-1 genome was derived by transfection. The modified cell line COS 10-11 contains one copy of the HIV-1 genome per cell as determined by Southern blot hybridization.

Lymphocyte samples (10^6 cells) from HIV-1-infected individuals were provided by Douglas D. Richman (Veterans Administration Medical Center, La Jolla, Calif.). These

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TABLE 1. Sequences of primers and probes

Primer or probe	Sequence (5′–3′)
5′ Primer S1	.....GGAACCCACTGCTTAAGCC
3′ Primer S2	.....GGTCTGAGGGATCTCTAG
Probe Eu^{3+}-S9	.....$(NH_2\text{-}C)_{35-40}$ACCAGAGTCACACAACAGAC GGGCA
Probe bio-S11	.....CACACTACTTGAAGCACTCAAGGCAAGC $(NH_2\text{-}C)$C

samples had been frozen in medium containing 40% fetal bovine serum and 10% dimethyl sulfoxide and were divided into aliquots of 2.5×10^5 cells, pelleted by centrifugation (1,000 × *g*, 5 min), and resuspended in 30 μl of H_2O prior to the PCR.

HIV-1 DNA reagents. A conserved 100-bp region of the HIV-1 long terminal repeat (nucleotides 506 to 605 of the HIVHXB2CG clone [22]) was selected for amplification. All oligonucleotides, including the full-length sense (+), named S32, and antisense (−), named S24, 100-mers were synthesized on a Gene Assembler (Pharmacia LKB Biotechnology) by following the instructions of the manufacturer. The sequences of the primers, S1 and S2, and the probes, Eu^{3+}-S9 and bio-S11, are given in Table 1. All oligonucleotides were purified on 10% polyacrylamide gels (19).

Genomic DNA was isolated from the COS 10-11 cell line by standard protocols (19), digested with *Eco*RI, and diluted in carrier DNA (salmon sperm DNA at 0.1 μg/μl) to be used as model DNA in our PCR protocol.

Synthesis and europium labeling of oligonucleotide S9. A diaminohexane-modified deoxycytidine phosphoramidite (27) was used in the DNA synthesis in a Gene Assembler (Pharmacia LKB Biotechnology) by using standard procedures. Good coupling yields, 98% or greater, were routinely obtained by using this reagent. The oligonucleotides containing the diaminohexane-modified deoxycytidine base (NH_2-C) were deprotected in 35% ammonia at 55°C overnight. Purification of these oligonucleotides was done on a 10% urea polyacrylamide gel (19). Bands corresponding to the approximate lengths of 60- to 65-mer were collected for elution and subsequent labeling. A population of the oligonucleotide S9 (Table 1) with approximately 35 to 40 NH_2-C's on the 5′ end was used for the Eu^{3+} labeling reaction. A 10-nmol amount of the oligonucleotide was dried and resuspended in 50 μl of H_2O. The pH was adjusted to 9.5 by adding Na_2CO_3 to a final concentration of 50 mM. A 4-μmol amount of Eu^{3+}-chelate W2014 (14, 27), which contains an isothiocyanate as its reactive group, was added. The reaction was allowed to proceed overnight at 4°C. The final product was purified on a Sephadex DNA grade G-50 (Pharmacia LKB Biotechnology) column (50 by 1 cm) by using 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5)–50 μM EDTA as the elution buffer. Fractions containing the labeled oligonucleotide product, Eu^{3+}-S9, were pooled. The content of Eu^{3+} was measured in a TR fluorometer (model 1230 Arcus; Wallac Oy), against a $EuCl_3$ standard. The oligonucleotide content was measured at 260 nm by using a correction factor for the absorption of Eu^{3+} chelate at that wavelength. A labeling degree of 20 Eu^{3+} chelates per oligonucleotide S9 was achieved.

Biotinylation of oligonucleotide S11. A 30-nmol amount of the oligonucleotide S11 containing a single diaminohexane-modified deoxycytidine at the 3′ end (Table 1) was dried and resuspended in 50 μl of H_2O. The pH was adjusted to 9.5 by adding Na_2CO_3 to a final concentration of 50 mM. A 1.5-μmol amount of biotin-amidocaproate *N*-hydroxysuccinimide ester (Sigma Chemical Co., St. Louis, Mo.) was added in dry *N*,*N*-dimethylformamide. The reaction was allowed to proceed for 2 h at room temperature, and the final product was separated from the excess biotin by running the reaction solution over an NAP-5 column and subsequently over an NAP-10 column (Pharmacia LKB Biotechnology) with 20 mM HEPES (pH 7.5)–50 μM EDTA as the elution buffer.

The biotinylated oligonucleotide was analyzed by fast protein liquid chromatography by use of a PEP RPC 5/5 (Pharmacia LKB Biotechnology) column. Buffer A consisted of 0.1 M triethylammonium acetate and 10% acetonitrile. Buffer B consisted of 0.1 M triethylammonium acetate and 30% acetonitrile. The gradient for buffer B was 0 to 100% in 50 min. As a control, the starting material was run under the same conditions. The reaction proceeded essentially to completion under the biotinylation conditions used above.

Biotinylation of BSA. A 10-mg amount of bovine serum albumin (BSA) was dissolved in 1 ml of 50 mM Na_2CO_3, and a 50 M excess of biotin-amidocaproate *N*-hydroxysuccinimide ester in 200 μl of dry *N*,*N*-dimethylformamide was added. The reaction was allowed to proceed for 3 h at room temperature. The final product was purified by running the reaction mixture twice over PD-10 columns (Pharmacia LKB Biotechnology) with 0.9% NaCl–20 mM HEPES (pH 7.5)–0.05% sodium azide as the eluent.

PCR amplification. The PCR was done in a mixture consisting of 10 mM Tris hydrochloride (pH 8.4), 2.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 0.2 mg of gelatin per ml, 50 mM NaCl, 0.5 μM (each) primer S1 and S2 (Table 1), and 1 U of *Taq* polymerase (The Perkin-Elmer Corp., Norwalk, Conn.) in a total volume of 100 μl. Samples were added in 10- (pure DNA) or 30-μl (cell samples) volumes. Cell samples (2.5×10^5 cells in H_2O) were heat lysed and centrifuged before the addition of the PCR mixture. The reaction tubes were incubated in a Thermocycler (Perkin-Elmer) by using the following program: 95°C, 50 s; 55°C, 2 min; 73°C, 2 min; 30 cycles.

ABC hybridization. A 10-μl volume of the amplification reaction mixture was analyzed in the ABC hybridization assay. The PCR sample was boiled for 8 min, cooled on ice, and centrifuged. A 100-μl volume of the hybridization solution, consisting of 500 mM NaCl, 50 mM HEPES (pH 7.5), 0.05% Tween-20, 100 μM EDTA, and 5 ng (each) of bio-S11 and Eu^{3+}-S9 probes per ml, was added. The hybridization assay was carried out for 1 h at 55°C. Collection of the formed hybrids was performed by transferring the hybridization mixture to individual streptavidin microtitration wells and incubating them with 100 μl of a collection buffer (1 M NaCl, 50 mM Tris hydrochloride [pH 7.75], 0.1% Tween-20, 0.5% BSA, 0.05% bovine globulin, 0.05% sodium azide) for 1 h at room temperature. The strips were washed six times with wash solution (10 mM Tris hydrochloride [pH 7.75], 0.9% NaCl, 0.1% Tween-20, 0.05% sodium azide) in an automated platewasher (model 1296-024; Wallac Oy). Enhancement solution (Wallac Oy) was added at 200 μl per well, and the strips were shaken for 25 min at room temperature. The fluorescence was measured in a model 1230 Arcus (Wallac Oy) TR fluorometer.

The streptavidin microtitration strips (catalog no. 4-77178; Nunc, Roskilde, Denmark) were prepared by coating with biotinylated BSA (100 ng per well) in 0.9% NaCl-50 mM K_2HPO_4 (pH 9.0)-0.05% sodium azide overnight at room temperature, followed by six washes with wash solution.

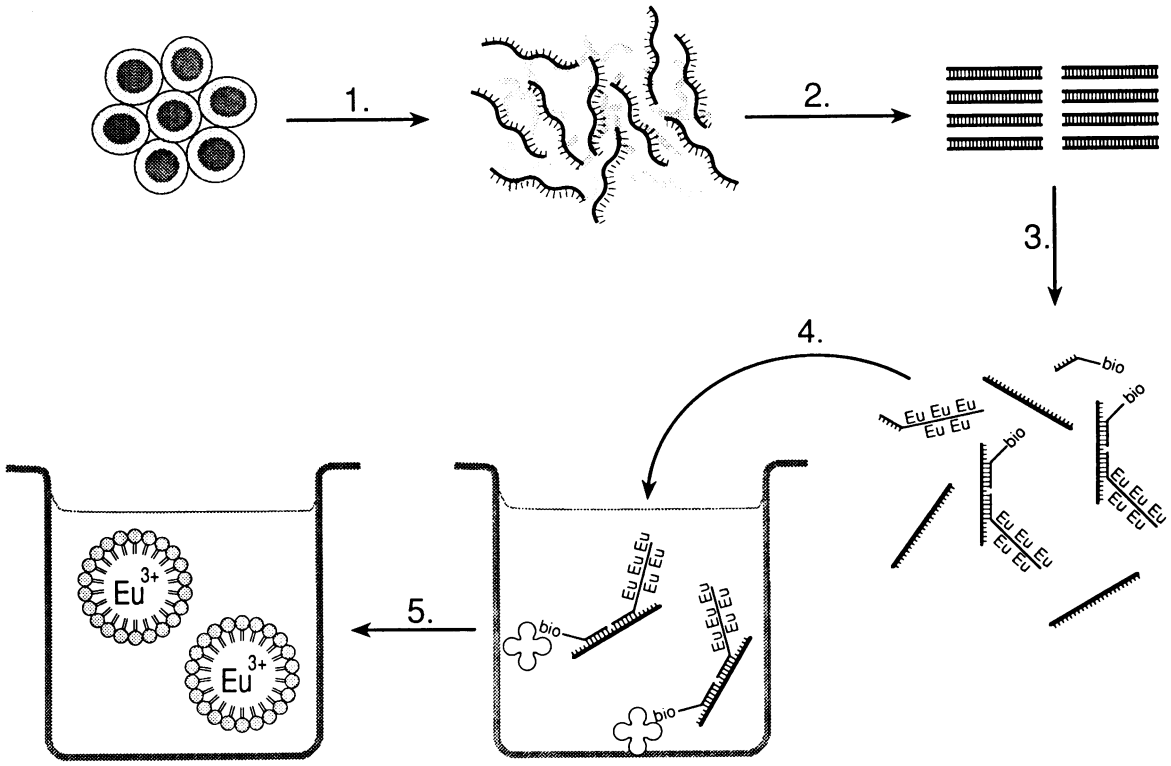

FIG. 1. Schematic of the assay. The cells are lysed (1.), and PCR is performed on the crude sample (2.). In the hybridization reaction two probes, one biotinylated and the other labeled with Eu^{3+}, complementary to the amplified fragment form sandwich hybrids with their target (3.). These hybrids are subsequently collected onto a streptavidin solid support (4.). The Eu^{3+} is dissociated from the bound Eu^{3+} probe by the enhancement solution and measured in a TR fluorometer (5.).

The biotinylated BSA surface was saturated with streptavidin (BRL, Gaithersburg, Md.) at 2 μg/ml in saturation buffer (50 mM Tris hydrochloride [pH 7.75], 0.1% Tween-20, 0.5% BSA, 0.05% bovine globulin, 0.05% sodium azide) by incubation for 3 h at room temperature and then washed six times as described above. These strips can be stored dry at 4°C for 1 year without significant loss of binding capacity.

RESULTS

The schematic for the assay presented in this study is outlined in Fig. 1.

ABC hybridization. The kinetic properties of the solution hybridization reaction were studied by using several approaches. First, the time needed for the hybridization reaction to come to completion was determined. Second, the effects of competition of the (−) strand with the probes for binding to the (+) strand on the kinetics of the reaction was studied. These are important considerations in the development of a quantitative assay. The question of strand competition is also interesting since the PCR can be modified to produce single-stranded products (9).

The hybridization reaction reaches the maximal signal at 1 to 3 h, depending on the amount of target DNA and whether the DNA is single or double stranded (Fig. 2). If single-stranded target is studied, the hybridization reaction seems to reach completion sooner, i.e., after 1 h, whereas with high amounts of double-stranded DNA, the reaction is complete after 3 h of hybridization. It is, however, not necessary to hybridize for times longer than 1 h to achieve sufficient sensitivity even if the target is double stranded. The slow

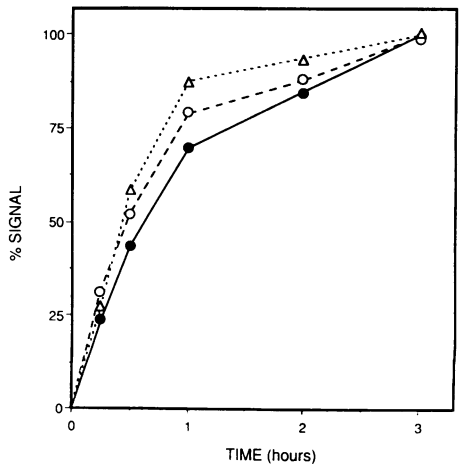

FIG. 2. Kinetics of hybridization reaction. The kinetics of the hybridization reaction was studied on single- and double-stranded DNA. Molecules (3×10^8) of the synthetic 100-mer S32 was used as the single-stranded target (△). PCR-amplified DNA fragments were used as double-stranded targets at two concentrations, 50 (○) and 1,000 (●) cell equivalents of initial target DNA, respectively. The hybridization was carried out for times ranging from 15 min to 3 h, otherwise the assay was performed as described in Materials and Methods. The 3-h time point is considered the 100% level. Results are given as the mean of triplicate samples.

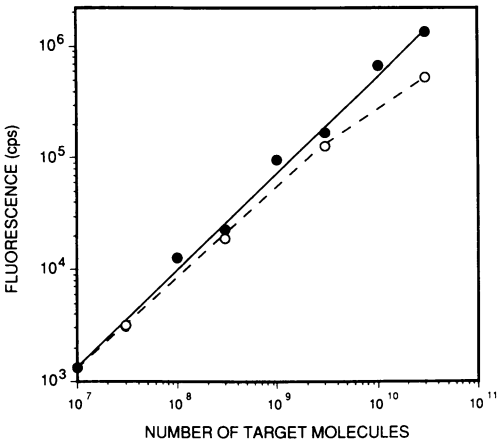

FIG. 3. Linearity of the ABC hybridization. Various amounts of the synthetic oligomer S32, mimicking a single-stranded target DNA, was analyzed in the hybridization assay as described in the text. To mimic the situation of double-stranded target DNA, equivalent amounts of the complementary S24 (−) synthetic 100-mer was added. Symbols: ●, single-stranded S32; ○, double-stranded DNA (equivalent amounts of S32 and S24). The results are given as the mean of triplicate samples. The background obtained from 0.1 μg of salmon sperm DNA was 650 ± 44 cps (±1 standard deviation).

hybridization rate is due to the low concentration of probes that is used in these assays. The (−) strand seems to affect the kinetics by which the probes hybridize successfully to the (+) strand by competing with the probes for hybridizing to the (+) strand. The competition of the (−) strand is concentration dependent.

The sensitivity that can be achieved in the ABC hybridization assay is 10^7 molecules of target DNA, when the cut-off was set at 2× background counts. The assay is linear with single-stranded DNA as target from 10^7 to 10^{10} molecules (Fig. 3). When double-stranded DNA is analyzed, the linear range is slightly shorter and the signals obtained are somewhat lower because of the competing (−) strand (Fig. 3).

Detection and quantification of minute amounts of HIV-1 DNA. A series of samples containing digested genomic DNA isolated from the COS 10-11 cell line, ranging from 5 to 1,000 cell equivalents of DNA diluted in salmon sperm DNA, which also was used as background DNA (1 μg per reaction), was amplified by PCR as described above. A 10-μl aliquot of each reaction mixture was tested in the ABC hybridization assay. We were able to reliably detect as few as 5 molecules of target HIV-1 DNA; at this level the mean signal was 3 times greater than the mean background (Fig. 4A). It is evident from our results that this PCR-based ABC hybridization assay is quantitative, since a reliable and reproducible standard curve can be generated. The assay curve is linear for from 5 to 500 copies of HIV-1 DNA (Fig. 4A). The variation in the signal levels generated from minute amounts of target molecules in the overall assay is typically 10 to 20% coefficient of variation. It can be estimated that the level of amplification achieved in our PCR protocol with 30 cycles is on the order of 10^8.

To further study the linearity aspect of this assay, we used the asymmetric PCR approach (9), thus producing single-stranded target molecules for the hybridization reaction. The PCR protocol was modified so that the primer S2 was set to limit the PCR. A concentration of 0.01 μM of S2 was used in the PCR mixture. To compensate for the lower efficiency in the amplification due to the low concentration of the limiting primer S2, it was necessary to increase the number of cycles from 30 to 35. The PCR samples from the asymmetric amplification scheme were not denatured before the hybridization reaction solution was added. The asymmetric PCR, when analyzed in the ABC hybridization format, generated a standard curve which was linear over the whole range tested, i.e., 5 to 1,000 molecules of HIV-1 DNA (Fig. 4B).

Cell samples. In a DNA probe assay, the sample pretreatment is crucial in that the DNA must be liberated from the cell nucleus and the histone complexes. Several investigators (8, 12, 23) have reported that simply boiling cells for the extraction of nucleic acid for the PCR is sufficient.

Crude cell samples, COS 10-11 cells in background lymphocytes (2.5×10^5 cells per reaction), were lysed in H_2O by heating and then tested in the PCR-ABC assay. A standard curve with 5 to 500 HIV-1-transfected COS 10-11 cells was generated (Fig. 5). The detection sensitivity with crude samples was 5 to 10 COS 10-11 cells, when 2× background was used as the cut-off.

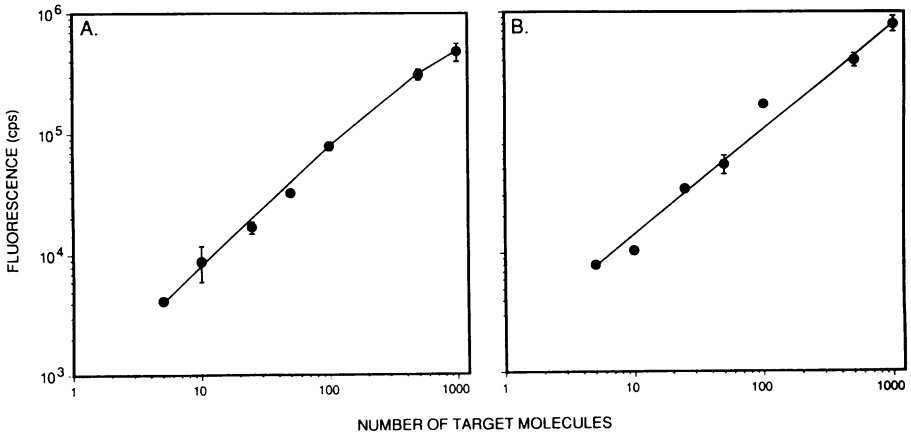

FIG. 4. Linearity of the PCR-based assay on HIV-1 DNA. PCR was run on DNA isolated from COS 10-11 cells, containing one copy of HIV-1 DNA per cell, at amounts ranging from 5 to 1,000 cell equivalents of genomic DNA from these cells. The PCRs, run symmetrically (A) and asymmetrically (B), and the subsequent ABC hybridization were performed as described in the text. The results are from duplicate tests; the bars indicate ±1 standard deviation. The background in both assays was 1,000 ± 120 cps (±1 standard deviation).

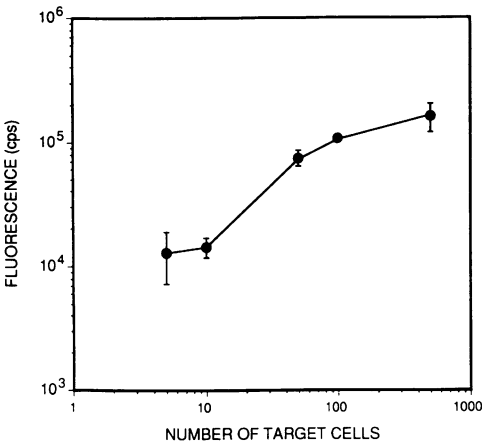

FIG. 5. PCR on crude cell samples. Various amounts of HIV-1 containing COS 10-11 cells, diluted in 2.5×10^5 noninfected lymphocytes, were analyzed in the PCR-based assay after hypotonic heat lysis of the cells. The test was run in triplicate as described in the text. Error bars indicate ±1 standard deviation. The background, obtained by testing 2.5×10^5 noninfected lymphocytes, in the assay was 5,760 ± 1,860 cps (±1 standard deviation).

We performed a series of studies with various amounts of background lymphocytes, and no decrease in signal levels due to an increase in cell debris up to 10^6 background cells could be observed. The background noise in the assay became somewhat elevated (2×10^3 to 8×10^3 cps) when crude cell samples were used. The increase in the background level was somewhat dependent on the amount of background cells added to the test (data not shown). (Extreme precautions [16] must be taken to avoid contamination and carry-over problems with a test at this level of sensitivity.) The variation between samples within one assay run was greater when crude samples were tested, typically 20 to 40% coefficient of variation, as compared with pure DNA samples (10 to 20% coefficient of variation).

Clinical samples. To test the utility of the developed assay on actual clinical specimens, 27 lymphocyte samples from HIV-1-infected individuals were tested. The samples were assayed at 2.5×10^5 cells per test. Three lymphocyte samples from seronegative healthy donors were used as negative controls. The samples from HIV-1-infected individuals all gave strongly positive signals in our assay, with the lowest positive signal more than sixfold higher than the negative controls (Table 2).

TABLE 2. Detection of HIV-1 nucleic acid from clinical samples

HIV-1 status	Patient no.	Mean signal[a]	% CV[b]	Assay result[c]
Uninfected	1	6,128	6.1	−
	2	5,978	29	−
	3	5,165	13	−
Infected	4	80,039	2.9	+
	5	103,163	4.4	+
	6	76,960	8.4	+
	7	129,937	40	+
	8	75,906	35	+
	9	38,140	33	+
	10	207,385	2	+
	11	169,997	0.65	+
	12	180,257	8.1	+
	13	198,737	14	+
	14	76,656	63	+
	15	39,658	21	+
	16	74,016	34	+
	17	124,549	7.3	+
	18	130,168	5.2	+
	19	144,830	12	+
	20	130,405	66	+
	21	38,926	8.3	+
	22	195,945	4.7	+
	23	170,266	0.75	+
	24	71,974	54	+
	25	153,518	0.72	+
	26	129,984	30	+
	27	208,418	1.2	+
	28	179,270	13	+
	29	190,476	6.7	+
	30	164,202	0.75	+

[a] A total of 2.5×10^5 cells were tested per test. Value is the mean of duplicates.
[b] CV, Coefficient of variation.
[c] The cut-off was determined as two times mean background ($2 \times 5,757$ cps).

DISCUSSION

The development of a novel, straightforward labeling method for direct coupling of europium chelates to oligonucleotides enabled us to initiate this study. The fact that the label to be detected is directly attached to the oligonucleotide probe simplifies the hybridization assay considerably. The directly labeled oligonucleotides facilitates other applications, such as the use of Eu^{3+}-labeled PCR primers. The sensitivity that is achieved with this labeling technology is enhanced by the fact that not one, but several, Eu^{3+} chelates can be coupled to one probe. When conventional fluorophores, such as fluorescein, are used, it is not advantageous to use multilabel procedures because of self quenching (10). However, when lanthanide chelates which have a long Stokes shift are used, the self quenching is less of a problem. Furthermore, since the Eu^{3+} is dissociated from the solid phase with the enhancement solution, no self quenching occurs with the detection methodology described here. The use of the NH_2-C simplifies the preparation of multiply labeled nonradioactive probes since commercially available supports can be used directly without modifications and the NH_2-C can be inserted at any position(s) in the sequence during synthesis according to standard protocols.

We are in the process of studying in more detail how the basic properties (melting temperature and hybridization kinetics, in particular) of the oligonucleotides are affected by the NH_2-C tail at either the 3′ or 5′ end. We are also investigating how the degree of Eu^{3+} labeling of the probes affects these properties. These studies will help to further improve the probe synthesis and labeling strategies.

In our study, particular interest was directed towards the linearity of the actual hybridization method. The use of microtitration strips as solid support affects the linearity of the assay, since the limited binding capacity of the streptavidin surface sets a limit for the amount of biotinylated probe that can be used to drive the hybridization to completion. Further improvements of the solid support would increase the binding capacity and, thus, enhance the quantitative nature of this assay. The solution hybridization technique presented here, utilizing two oligonucleotide probes competing for hybridization to the target (+) strand with a full-length complementary (−) strand, results in a narrow linear range of the assay. To avoid complex hybridization reaction

products, one could use the asymmetric PCR, which produces a single-stranded amplification product. As we have shown here, this approach works and, indeed, results in a slightly wider linear dose response in the assay.

The sensitivity of the post-PCR detection method is generally not considered important, given the amplification power of the PCR. TR fluorescence as a sensitive detection method can potentially reduce the need for amplification and therefore also reduce the risk for contamination. In addition, the efficiency of the PCR drops when the number of cycles is increased, and therefore the sample variation is less of a problem with fewer cycles. The sensitivity achieved in the ABC hybridization method based on Eu^{3+}- and biotin-labeled oligonucleotides is 10^7 molecules of target DNA. Combined with the PCR, which on this particular HIV-1 long terminal repeat fragment was optimized to facilitate an amplification factor of 10^8 in 30 cycles, an overall sensitivity of five molecules of target DNA initially added to the test is easily achieved, even if only 1/10 of the amplified product is analyzed in the hybridization reaction. If desired, the overall sensitivity of this assay could potentially be increased by running 50-μl PCRs and testing the whole amplification solution in the ABC hybridization assay.

The HIV-1 application chosen for the feasibility study of this assay is an extremely interesting and challenging one. We were able to successfully detect HIV-1 proviral DNA from lymphocyte samples isolated from HIV-1-infected individuals. It is evident that, with this assay, one should be able not only to detect HIV-1 but to quantitate the virus load in an infected individual. With ongoing trials with antiviral drugs, assays with these characteristics will be increasingly interesting.

By including a cDNA reaction step prior to the PCR, it is possible to detect expressed HIV-1 RNA from cells or viral RNA contained by virus particles, rather than proviral DNA, and thus quantitate the degree of expression of HIV-1 RNA or even the number of free viruses in plasma. Measuring viral RNA might prove prognostic, since the degree of HIV-1 viremia has been reported as an indicator of the disease state of infected individuals (11).

In this study we have developed a DNA probe-based assay which is rapid, user friendly, sensitive, and accurate. It does not require radioactive labels nor does it require electrophoresis. The results are given as numbers and are not determined by staining or autoradiography. Finally, the design of the assay can easily be modified for automation, since the format that is used is the microtitration format commonly used in routine laboratory analyses. It is evident from our experience that unless a method for eliminating PCR product contamination is developed, a closed automated system must be designed to prevent the amplified DNA fragment from contaminating the laboratory when in routine use in clinical laboratories.

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