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Detection of Human Immunodeficiency Virus Type 1 Proviral DNA by PCR Using an Electrochemiluminescence-Tagged Probe

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We have developed a rapid, pseudohomogeneous assay for the detection of PCR amplicons, based on the use of electrochemiluminescence generated from a Tris-bipyridine ruthenium(II) label. PCR amplification of highly conserved human immunodeficiency virus type 1 (HIV-1) *gag* gene sequences was performed with SK38 and SK39 primers, the latter of which was 5 biotinylated. Post-PCR reaction mixtures were combined with 10^{12} copies of the SK19 probe–Tris-bipyridine ruthenium(II) conjugate, denatured by heating at 100 C for 5 min, and hybridized at 55 C for an additional 15 min. Hybridization to the biotinylated strand of the amplified DNA was determined by the addition of streptavidin-conjugated magnetic particles and analyzed by using an Origen-1 electrochemiluminescence analyzer. Our results demonstrated a sensitivity of fewer than five copies of HIV-1 (pre-PCR), by using either purified plasmid DNA containing one complete copy of the HIV-1 cDNA genome or lysed, proteinase K-treated 8E5 cells as the starting material. In an evaluation of actual clinical specimens (peripheral blood monocytes from both healthy and HIV-1-infected children), the electrochemiluminescent detection assay correlated 100% with both our standard method (solution hybridization with a radiolabeled probe followed by polyacrylamide gel electrophoresis [PAGE] and autoradiography) and a commercial method (Roche Amplicor). The electrochemiluminescent method was substantially easier to perform than either the PAGE or microtiter plate assays and was considerable faster to perform than either of these alternative formats.

Since the identification of human immunodeficiency virus type 1 (HIV-1) as the etiologic agent of AIDS in 1983 (2, 7), a variety of methods have been developed for the diagnosis of HIV-1 infections. Adult HIV-1 infections are most often diagnosed indirectly by serologic assays, which detect the presence of antibodies to one or more viral proteins (4, 18). These assays are highly reliable, but false-positive or indeterminate results do occasionally occur (16). Serological testing can also miss recently infected individuals who have not yet seroconverted (18). Immunoglobulin G antibody detection assays are inappropriate for determining the infection status of infants born to HIV-1-positive mothers, because of the confounding presence of maternal antibodies (18).

Assays for the direct detection of HIV-1 fall into three categories: (i) viral culture, (ii) viral antigen detection, and (iii) detection of viral RNA or proviral DNA. While culture techniques have been greatly improved, the procedures are laborious, time consuming, and costly and require the handling of significant amounts of an infectious agent. First generation immunoassays for the detection of viral proteins, specifically, the p24 core antigen, can be insensitive because of the formation of immune complexes between p24 and host antibodies (3). The sensitivity of p24 antigen assays has been significantly enhanced by the development of immune complex dissociation procedures (5, 14, 19).

Nucleic acid probe assays for HIV-1, based on PCR amplification, have been demonstrated to be highly sensitive and specific (1, 9). PCR assays have been very useful for resolving the infection status of individuals with an indeterminate serological status (8, 10) and for determining the infection status of infants born to HIV-1-infected women (17, 20). An early criticism of PCR-based assays was that they were too complicated for routine use in clinical laboratories. In addition, analysis of PCR products typically relied on the use of radiolabeled oligonucleotide probes. In the past few years, approaches have been reported for simplifying the analysis of PCR amplification products. The main focus of these efforts has been the development of nonradioactive assay formats, mostly by using chemiluminescent or enzyme labels or a combination of the two, to obtain a desired level of sensitivity (21, 22).

Recently, Kenten et al. (12) have described the utility of a novel electrochemiluminescent (ECL) label, Tris-bipyridine ruthenium(II) complex, in both immuno- and nucleic acid probe assays. The use of ECL label has several advantages over other nonradioisotopic methods. Unlike chemiluminescent, fluorometric, or colorimetric methods, which produce signals via enzyme-mediated hydrolysis of a substrate, resulting in the production of a signal, the ECL reporter group is detected directly by using a dedicated instrument, the Origen-I (IGEN Inc., Rockville, Md.) analyzer. This results in a significant simplification of the detection component of the PCR assay, which further translates into savings in both reagent and labor costs.

In this paper, we describe a simple and rapid method for the detection of PCR products based on oligonucleotide solution hybridization by using an ECL-labeled oligonucleotide probe for the detection of HIV-1 proviral DNA sequences in peripheral blood mononuclear cells (PBMCs). The performance of our ECL probe assay, compared with both the classical method, oligonucleotide solution hybridization with radiolabeled probes and polyacrylamide gel electrophoresis (PAGE), and a commercially available PCR method (Roche Amplicor HIV-1 PCR assay), is also discussed.

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MATERIALS AND METHODS

Oligonucleotides. HIV-1 *gag* gene-specific PCR primers, SK38 and 5′-biotinylated SK39, and the detection probe SK19 (15) were synthesized by Genosys Biotechnologies Inc. (The Woodlands, Tex.). The probe was synthesized with a 5′-terminal amine to allow coupling to the ECL label. Both SK39-biotin and SK19-amine were purified by high-performance liquid chromatography by the manufacturer.

Labeling of oligonucleotides. Unmodified SK19 was radiolabeled with [γ-^{32}P] ATP and T4 polynucleotide kinase by established methods (13). The ECL label was attached to the modified SK19 probe via the 5′-terminal amino group as follows: the oligonucleotide (0.1 μmol) in 100 μl of phosphate-buffered saline (PBS) was reacted with 5 μmol of an *N*-hydroxy-succinimide ester of Tris-bipyridine ruthenium(II) dihexafluorophosphate (IGEN Inc.) in 400 μl of dimethyl sulfoxide and held overnight at room temperature in the dark. The oligonucleotide was recovered from the labeling reaction mixture by precipitation with ethanol, dissolved in 1 ml of TE buffer (10 mM Tris-HCl [pH 8.0], 2 mM EDTA), and stored in 50-μl aliquots at −20°C.

Specimen collection. Except where noted, the majority of specimens employed in this study were obtained by venipuncture from children between the ages of 2 weeks and 12 years. Blood was collected in Vacutainer tubes (Becton Dickinson Vacutainer Systems, Rutherford, N.J.) containing acid citrate dextrose and stored at room temperature for not more than 18 to 24 h prior to analysis.

HIV-1 cultures. Viral microculture assays for the detection of HIV-1 in the PBMCs of HIV-1-infected individuals were performed as described by Erice et al. (6).

PCR. PCR assays, developed in-house, were performed essentially as described by Kellogg and Kwok (11). PBMCs were separated by Ficoll-Hypaque density gradient centrifugation and washed three times in PBS. One million cells were removed to a fresh 1.5-ml microcentrifuge tube and centrifuged at 400 × *g* for 10 min. The supernatant was removed, and the cells were suspended in 250 μl of lysis buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 0.45% Nonidet P-40, 0.45% Tween 20, 10 μg of proteinase K per ml). The lysates were incubated at 60°C for 1 h and then at 100°C for 10 min to inactivate the proteinase K. A 50-μl sample of each specimen was transferred to a thin-walled, 200-μl MicroAmp (Perkin Elmer, Norwalk, Conn.) tube containing 50 μl of amplification master mix (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 500 μM each deoxynucleoside triphosphate, 500 μM [each] SK38 and SK39-biotin primers, 2.5 U of Ampli*Taq* DNA polymerase [Perkin Elmer]). Amplification was performed in a GeneAmp System 9600 (Perkin Elmer) thermocycler by using the following program: 5 cycles at 95°C for 10 s, 55°C for 10 s, and 72°C for 10 s, followed by 30 cycles at 90, 60, and 72°C each for 10 s. At the end of the program, the tubes were held at 4°C.

Processing of blood specimens and amplification of HIV-1 proviral DNA sequences by using the Amplicor HIV-1 PCR kit (Roche Molecular Systems, Branchburg, N.J.) and subsequent analysis using the Amplicor PCR detection kit were done according to the manufacturer's instructions.

Hybridization and detection of PCR products. Detection of PCR products by oligomer solution hybridization (OSH) with a ^{32}P-labeled SK19 probe and PAGE was done as described by Kellogg and Kwok (11) (Fig. 1A). ECL analysis of PCRs with the ECL-labeled probe was done as follows (Fig. 2B). The ECL-labeled SK19 oligonucleotide was diluted to 10^{11} copies of probe per μl in hybridization buffer (1× SSC [0.15 M NaCl plus 0.015 M sodium citrate] 0.1% sodium dodecyl sulfate, 5× Denhardt's solution). To a fresh, 0.2-ml microcentrifuge tube, 10 μl each of the diluted probe, tRNA solution (100 μg/ml in hybridization buffer), and the PCR mixture being analyzed was added. Incubation of the tubes at 100°C for 5 min to denature the double-stranded PCR products was followed by a second incubation at 55°C for 15 min to permit annealing of the probe to the target DNA. Each hybridization mixture was transferred to a separate polypropylene tube (13 by 75 mm) containing 50 μl of a 400-μg/ml suspension of streptavidin-coated magnetic particles (Dynal M280; Dynal Inc., Long Island, N.Y.) in Origen assay buffer (OAB; IGEN Inc.) and vortexed on the Origen-I (IGEN Inc.) analyzer at 88 rpm for 15 min at room temperature. Subsequent separation and washing steps and ECL measurements were performed by the Origen-I analyzer as previously described (12).

Analysis of Amplicor PCRs by using the Amplicor HIV-1 PCR detection kit was performed as directed by the manufacturer's instructions.

RESULTS

The initial experiments were designed to compare the sensitivities of the standard OSH-PAGE assay (Fig. 1A) and the ECL detection method (Fig. 1B). PCR mixtures were assembled by using healthy donor PBMCs spiked with increasing amounts of 8E5 cells (ATCC CRL 8993, which carry one proviral copy of HIV-1 per cell). The results (Fig. 2) demonstrate equivalent sensitivities of both assays at a level of 10 to 12 input copies of HIV-1 proviral DNA (preamplification). HIV-1-specific amplified DNA was detected in the PCR resulting from two input copies of proviral DNA only by the ECL assay, illustrating increased sensitivity compared with that of OSH-PAGE. An additional and important observation was a nearly linear increase in the ECL signal concomitant with increasing amounts of preamplification levels of HIV-1 proviral DNA (Fig. 2B). This correlation between ECL signal strength and target copy number suggests that the development of a quantitative HIV-1 PCR assay should be possible.

To compare the performance of OSH-PAGE and OSH-ECL detection with specimens from actual patients, PCR amplifications were performed on PBMCs isolated from 14 blinded blood specimens. Eight specimens were obtained from patients known to be infected by HIV-1 as determined by viral culture; the remaining six were obtained from healthy, HIV-1-seronegative individuals. The confirmed HIV-1-positive statuses of six patients were detected by autoradiographic analysis resulting from OSH-PAGE (Fig. 3A); all eight were positive by ECL probe analysis (Fig. 3B). The two specimens that were missed by OSH-PAGE gave very low, but significant ECL signals, suggestive of a low viral load for both patients. All six healthy controls were negative for the presence of HIV-1 proviral DNA by both methods.

A direct comparison of our ECL-based PCR assay with a commercial, microtiter plate-based method (Roche Amplicor) was performed with 39 specimens obtained from adult patients, 29 of whom were infected with HIV-1 (confirmed by HIV-1 virus culture) and 10 of whom were HIV-1 seronegative and culture negative. All specimens were analyzed in duplicate by each method. The results of this comparison are shown in Table 1. One false-negative result was obtained with the Amplicor system. The same specimen was positive (in both replicates) by OSH-ECL. As in the case of the OSH-PAGE false-negative specimens, both ECL values for this specimen were very low, again suggesting that the viral load was very low.

An additional 60 blinded specimens were processed for PCR as described above, and the amplifications products were analyzed by OSH-ECL. Thirty-nine specimens were drawn from children with confirmed HIV-1 infections (by HIV-1 culture), and the remaining 21 were from HIV-1-culture-negative subjects. As shown in Fig. 4, the numerical ECL values for the negative specimens were tightly clustered, ranging from 118 to 206 ECL units, with a mean of 162 ECL units. The cutoff value for positive specimens was established at 218.8 ECL units (the mean of the negative specimens plus 2 standard deviations). ECL values for the PCRs with the 39 known positive specimens ranged from 807 to 71,437 ECL units; there was no overlap in ECL values among any of the positive and negative specimens.

DISCUSSION

Both noncommercial PCR assays (illustrated in Fig. 1) used in this study are based on the hybridization, in solution, of a labeled oligonucleotide probe to a complementary sequence present on one of the strands of the amplified DNA target. By the OSH-PAGE method, hybridization reaction mixtures are analyzed by electrophoresis through a polyacrylamide gel; the hybridized probe migrates through the gel matrix more slowly than do the unbound, single-stranded molecules. Autoradiography is performed to detect the location of the probe in the gel. Although this method enjoys wide use for detecting PCR-amplified DNA, it has several drawbacks. First, OSH-PAGE relies on the use of radioactively labeled probes. The radioac-

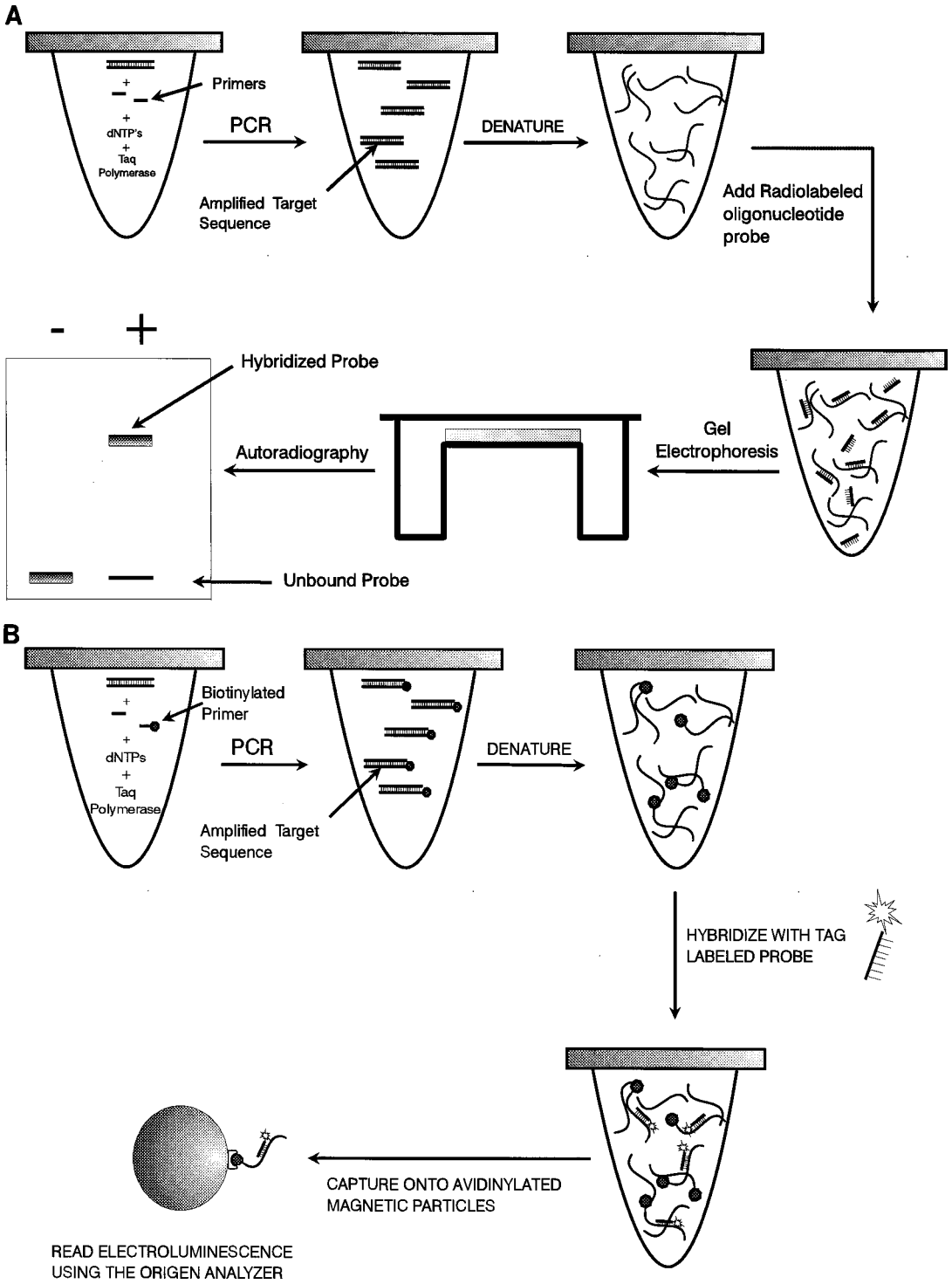

FIG. 1. Schematic representations of the standard OSH-PAGE (A) and OSH-ECL (B) PCR protocols. dNTPs, deoxynucleoside triphosphates.

tive tag has a short half-life, requiring the frequent preparation of fresh probe. There are the additional problems of storage and disposal of radioactive waste materials. Secondly, the preparation of polyacrylamide gels and loading of samples require personnel with a fairly high level of training. The use of commercially available, precast gels partly simplifies the procedure but adds appreciably to the overall cost of the assay. Sample throughput is also limited, depending on the number and sample capacity of electrophoresis chambers present in the laboratory.

Nonisotopic methods for the analysis of PCR products have been described previously (21, 22). Two of the most commonly used methods are PAGE-Southern blot hybridization and dot blot hybridization. Both techniques are very labor intensive

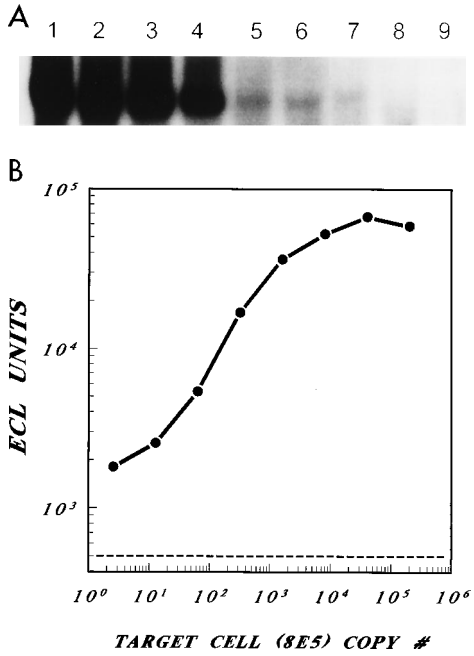

FIG. 2. Detection of PCR-amplified HIV-1 proviral DNA from 8E5 cells by OSH/PAGE (A) and OSH-ECL (B) analyses. (A) The HIV-1 DNA copy numbers in lanes 1 to 9 were 1×10^6, 2×10^5, 4×10^4, 1,600, 320, 64, 13, 2.5, and 0, respectively. The dotted line represents the negative cutoff value (the average of three negative controls plus 2 standard deviations).

and time consuming and require a high level of technical training. The use of radioactive probes has largely been replaced by enzyme-labeled probes and colorimetric or chemiluminescent substrates. Major benefits of enzyme labeling have been the increase in the long-term stability of the probes and eliminating the above-mentioned problems associated with using radioactive isotopes. However, both colorimetric and chemiluminescent detection require additional reagents and manipulations, adding to both the cost and complexity of these assays.

Further simplification of PCR analyses has been achieved through the use of microtiter plate assays, utilizing enzyme-tagged probes, and colorimetric or chemiluminescent and fluorometric substrates (21, 22). Enzyme immunoassays based on similar formats have been employed in clinical laboratories for several years; therefore, the adaptation of nucleic acid detection to microtiter plate methods has greatly reduced the need for retraining technologists. Microtiter plate-based assays, however, require multiple rounds of reagent additions, incubations, and washing steps, requiring a significant amount of the technologist's time and effort.

The OSH-ECL detection method described here is similar to OSH-PAGE in that the oligonucleotide probe is hybridized to the target DNA in solution; however, the target DNA strand is labeled at the 5′ end with biotin, and the probe is conjugated with the ECL label. Probe molecules hybridized to the biotinylated target DNA are captured onto magnetic particles coated with streptavidin; detection is accomplished by using an automated instrument, the Origen-I analyzer. Unlike radiolabeled probes, ECL-labeled oligonucleotides can be stored long-term (for at least 12 months) at −20°C, eliminating the need to prepare small amounts of freshly labeled probe every 2 weeks. ECL detection of PCR products is significantly simpler and faster than OSH-PAGE. The time to result for the latter procedure is approximately 4 to 5 h. The first results from the Origen-I analyzer are available within 30 to 45 min; subsequent specimen results follow at 1-min intervals (50-sample maximum). Simplification of the procedure results from the use of the Origen-I analyzer, which automatically performs all of the mixing, washing, and detection steps subsequent to placing the tubes containing the hybridization reaction mixtures and streptavidinylated magnetic particles onto the instrument.

Our results demonstrate that the sensitivity of PCR detection assays using ECL-labeled probes is equal to, if not greater than, that of OSH-PAGE. The linearity of the ECL results shown in Fig. 2 strongly suggest that a quantitative assay is feasible. Work is in progress to develop an ECL-based quantitative HIV-1 PCR assay.

Our preliminary data, comparing the OSH-ECL method with a commercially available HIV-1 PCR microtiter plate assay (Roche Amplicor), again demonstrated a degree of sensitivity equivalent to, if not greater than, that of the Amplicor

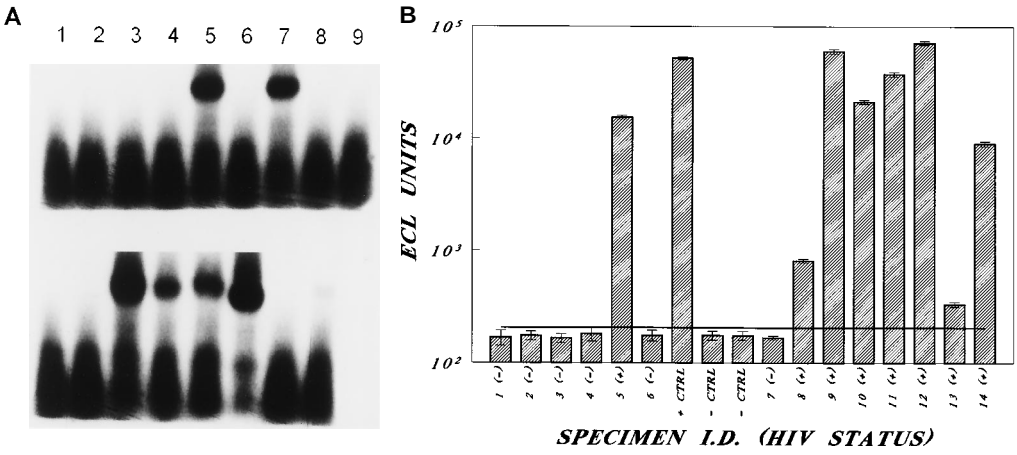

FIG. 3. PCR amplification and analysis of HIV-1 proviral DNA in PBMCs from HIV-1-infected and uninfected individuals. (A) Autoradiogram resulting from OSH-PAGE analysis. The samples in lanes 1 to 9 (top panel) and 1 to 8 (bottom panel) correspond in order to those in panel B. (B) OSH-ECL analysis. Annotations of plus and minus refer to the HIV-1 infection status of the subject. Negative controls specimens consisted of lysates prepared from H9 cells; an 8E5 cell lysate was used for the positive control (CTRL). The solid line represents the negative cutoff value (the average of three negative controls plus 2 standard deviations).

TABLE 1. Comparison of Roche Amplicor and OSH-ECL PCR detection methods[a]

Specimen no. (HIV-1 infection status)	Roche Amplicor		OSH-ECL	
	OD[b]	HIV result	ECL units	HIV result
1 (+)	4.075	+	54,799	+
2 (+)	4.200	+	54,139	+
3 (+)	4.021	+	33,833	+
4 (+)	4.200	+	53,293	+
5 (+)	4.200	+	27,375	+
6 (+)	4.175	+	40,029	+
7 (+)	4.011	+	29,761	+
8 (+)	4.128	+	35,453	+
9 (+)	4.200	+	14,110	+
10 (+)	4.134	+	14,148	+
11 (+)	3.312	+	38,310	+
12 (+)	0.070	−	2,017	+
13 (+)	4.200	+	52,968	+
14 (+)	4.027	+	59,914	+
15 (−)	0.092	−	250	−
16 (−)	0.077	−	253	−
17 (+)	3.958	+	7,377	+
18 (+)	4.200	+	8,025	+
19 (+)	4.200	+	10,482	+
20 (+)	4.200	+	7,823	+
21 (−)	0.057	−	150	−
22 (−)	0.074	−	141	−
23 (+)	4.161	+	11,338	+
24 (+)	4.200	+	12,064	+
25 (+)	4.200	+	4,823	+
26 (+)	4.178	+	15,914	+
27 (+)	2.498	+	4,190	+
28 (+)	4.191	+	8,954	+
29 (+)	4.200	+	12,830	+
30 (+)	4.200	+	13,468	+
31 (+)	4.200	+	13,023	+
32 (−)	0.057	−	123	−
33 (+)	3.798	+	1,244	+
34 (+)	3.388	+	9,988	+
35 (−)	0.052	−	151	−
36 (−)	0.049	−	98	−
37 (−)	0.074	−	139	−
38 (−)	0.051	−	119	−
39 (−)	0.055	−	130	−
Control				
2 copy	1.864	+	1,715	+
5 copy	3.856	+	6,028	+
Negative	0.075	−	166	−

[a] The cutoff values were 0.35 and 265 for a positive result by the Amplicor and OSH-ECL methods, respectively.
[b] OD, optical density.

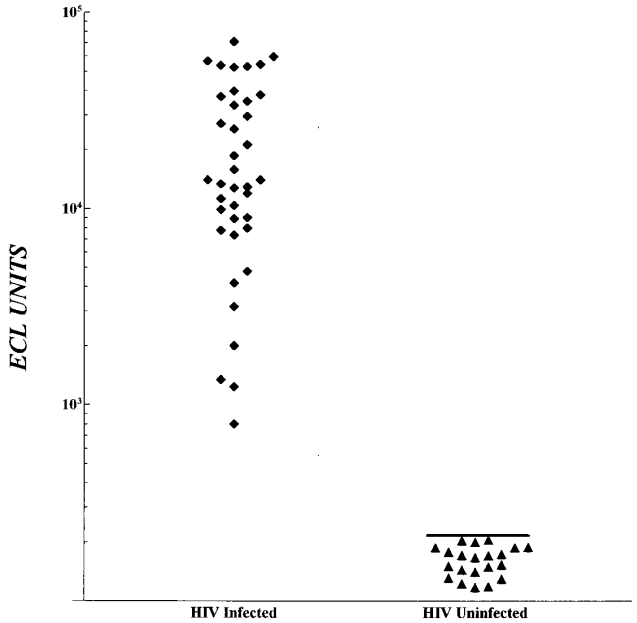

FIG. 4. OSH-ECL analysis of PCRs with PBMCs from 60 blinded specimens.

method. In our laboratory, OSH-ECL was considerably faster to perform, with significantly fewer manual operations. In addition, the ECL method was significantly less expensive on a per-test basis (~$13.00 per patient's result) than either the OSH-PAGE (~$24.00) or the Amplicor (~$18.00) assay. It should be noted that the cost per test of the OSH-ECL assay does not factor in the cost of the Origen-I analyzer.

In summary, we have developed a rapid and simplified semiautomated method for the detection of PCR amplification products. Our method employs a specific, ruthenium(II) bipyridyl-labeled oligonucleotide probe, complementary to the nucleic acid sequence amplified by using a 5′-biotinylated PCR primer. The assay format allows detection of the ECL signal on the surface of streptavidin-coated magnetic particles by using a semiautomated instrument, the Origen-I analyzer. We are now in the process of adapting OSH-ECL technology to other infectious disease PCR assays currently performed in our laboratory.

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