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Detection of Human Immunodeficiency Virus Type 1 DNA by Polymerase Chain Reaction Amplification and Capture Hybridization in Microtiter Wells

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We developed an improved microtiter-based assay for the detection of polymerase chain reaction (PCR)-amplified DNA sequences. The synthetic DNA sequences used to prime the PCR were labeled with biotin at their 5′ ends so that the specific PCR product was labeled with biotin. Following amplification, an aliquot of the PCR product was denatured and hybridized to a capture DNA sequence immobilized in a microtiter well. The capture sequence was complementary to a portion of the sequence between the primers, so that only extended primers were captured. The captured PCR product was detected colorimetrically by using a streptavidin-peroxidase conjugate and tetramethylbenzidine substrate.

Diagnosis of human immunodeficiency virus type 1 (HIV-1) infection usually involves the indirect detection of specific antiviral antibodies. Of the various direct methods available, amplification of HIV-1 DNA by the polymerase chain reaction (PCR) has been shown to be the most sensitive and definitive means of confirming HIV-1 infection (1–3, 6, 8, 9). Our goal in applying the PCR technology to the detection of HIV-1 has been to simplify the detection of the PCR product. In this report, we describe an improved method for the detection of PCR products which we call a capture hybridization assay. This assay is an improvement over our sandwich hybridization assay (4, 5) because is has an inherently lower background and is easier to perform. These improvements are a result of eliminating the labeled detection probe. Instead of a detection probe, the PCR product itself is labeled during the PCR, and it serves as both the sample and detection probe.

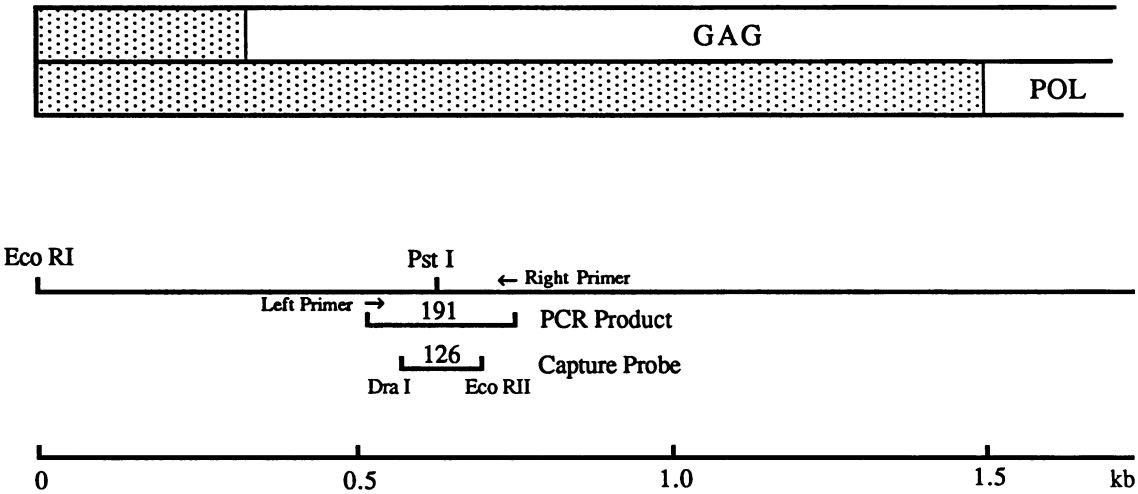

FIG. 1. Map of the HIV-1 genome showing the amplified region and the location of the capture probe sequence. The HIV-1 *gag* primers were used to prepare preparative amounts of the 191-bp PCR product from a template of pBH10 DNA. Following purification by gel electrophoresis, the product was digested with *Dra*I and *Eco*RII to yield a 126-bp fragment with endpoints at positions 885 and 1010 (7). The gel-purified restriction fragment was blunt-ended and cloned into a Bluescript KS$^-$ vector (Stratagene).

The region of the HIV-1 genome that was amplified by PCR is illustrated in Fig. 1. A region of the *gag* gene, in the p24-coding region, was chosen for amplification. This region is highly conserved in all HIV-1 isolates that have been

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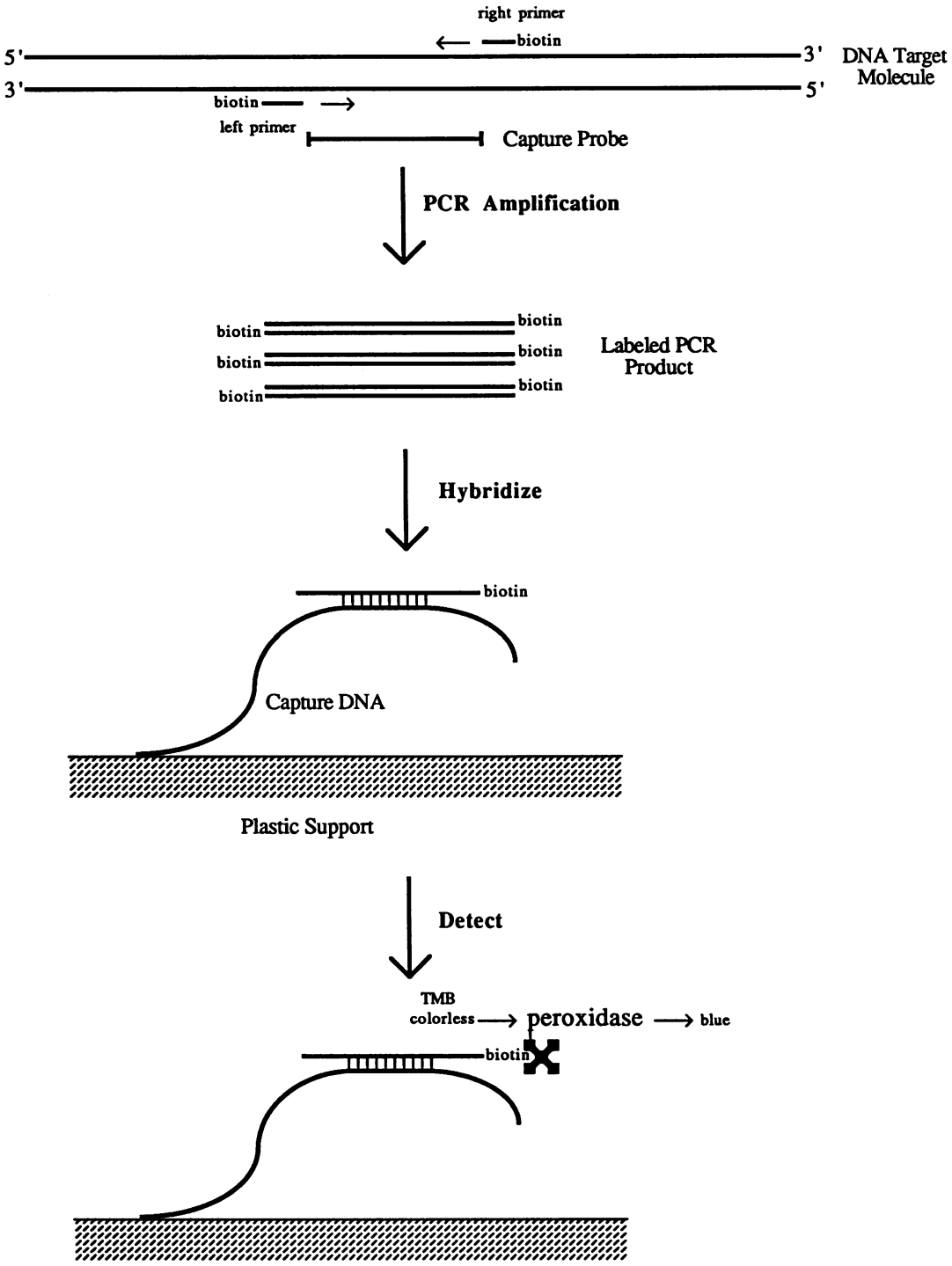

FIG. 2. Schematic diagram of the HIV-1 capture hybridization assay. The biotinylated PCR primers were extended, in the presence of target DNA, by DNA polymerase. The biotin-labeled amplification product was captured by hybridization to an immobilized capture probe and detected by incubation with streptavidin-peroxidase and a colorimetric substrate.

sequenced. Our primers amplify a 191-bp region surrounding the *Pst*I site in the *gag* gene. The capture probe is a 126-bp sequence contained within the 191-bp sequence, but it does not overlap the primer sequences. It is critical to avoid homology between the primers and capture DNA; otherwise, the labeled primers hybridize directly to the capture DNA and generate false-positive signals.

Extension of the labeled primers and detection of the PCR product are illustrated in Fig. 2. In the presence of specific target DNA, the primers are extended by *Taq* DNA polymerase to contain sequences homologous to the capture probe. The resulting biotin-labeled PCR product was immobilized by capture hybridization in microtiter wells. After hybridization, the bound PCR product strands were detected

by using enzyme-conjugated streptavidin and a colorimetric substrate.

The cutoff value for the capture assay was determined by testing a total of 30 samples from normal blood donors that were negative for HIV-1 DNA by PCR and Southern hybridization (data not shown) and calculating the mean and standard deviation (0.039 ± 0.034 absorbance units). The value of the mean plus 3 standard deviations was 0.14 absorbance units, and this was selected as the cutoff value for positive samples. All DNA samples were amplified by using 35 temperature cycles in order to detect weak HIV-1 samples reliably. The resulting degree of amplification was about 10^7-fold.

DNA extraction, primers, and PCR conditions were those described previously (4), except that the primers were labeled with primary amino groups at their 5′ ends by using Aminolink II (Applied Biosystems). The 5′ amino groups were covalently modified with biotin as follows. Sixty micrograms of the amino group-labeled primer was combined with 500 μg of NHS–long-chain biotin (Pierce) in 0.1 M $NaHCO_3$ (pH 9.0) and allowed to react for 2 h at room temperature. The biotin-labeled primer was recovered by ethanol precipitation. The human β-globin sequences from each sample were amplified in a separate reaction as described previously (5) to serve as an internal control. In order to determine the sensitivity of the capture assay, total DNA was extracted from HIV-1-infected cultured cells, and serial dilutions were prepared. An aliquot of each dilution was amplified by PCR, and the reaction products were analyzed by capture and Southern hybridization. Southern transfers and filter hybridization conditions were as described previously (5). For detection by capture hybridization, a 10-μl aliquot of each PCR product was mixed with 2 μl of carrier DNA (20 μg), and the mixture was denatured by boiling. One hundred microliters of hybridization buffer was added to the denatured sample, and this was transferred to microtiter wells coated with capture DNA as described elsewhere (5). Samples were hybridized for 4 h at 42°C. After hybridization, the wells were washed four times with 200 μl of 1× TBS (25 mM Tris [pH 7.4], 3 mM KCl, 137 mM NaCl) at room temperature. The detection conditions were those described previously (5).

The PCRs contained from 40,000 to 0.4 copies of target HIV-1 DNA before amplification. By using the cutoff value of 0.14, the capture assay was able to detect as few as four target molecules (A_{450} = 0.24), and this was equivalent to the Southern blot sensitivity. Thus, the detection range of the assay was 4 to 40,000 initial target molecules.

Next, a total of 41 clinical samples were assayed for HIV-1 DNA by both capture and Southern hybridization. DNA was extracted from cultured cells or peripheral blood lymphocytes of patients at risk for HIV-1 or human T-lymphotropic virus type 1 (HTLV-1) infection as described previously (4). Patient blood samples were obtained from Robert Biggar of the National Cancer Institute (samples were from high-risk homosexuals and patients with AIDS); Winston Frederick of Howard University Medical School (infants of seropositive mothers); James Shih of the Blood Bank, National Institutes of Health (HIV-1 culture positive); and Richard Egan of DuPont (HTLV-I-seropositive individuals). The DNA samples were amplified by the PCR, and the HIV-1 *gag* product was detected by capture and Southern hybridization. The HTLV-I-seropositive samples were included as negative controls. By using the cutoff value of 0.14, there was complete agreement between the capture hybridization and Southern hybridization assays: a total of 24 samples were positive by both Southern hybridization and the capture hybridization assay, and 17 samples were negative by both assays.

We have described a new nonradioactive assay format for the direct detection of specific HIV-1 PCR products. This capture format exhibits a lower background signal than that of the sandwich format, because the excess sandwich probe present in every well tends to bind nonspecifically to each well. When identical PCR samples were tested by the capture format and sandwich formats, the capture format resulted in a signal-to-noise ratio 5 to 10 times greater than that for the sandwich format (data not shown). Thus, weakly positive samples can be detected more reliably by the capture format.

The use of biotinylated primers during PCR amplification has been described previously (11). In that report, the biotin was not used to detect the product directly but to capture product-probe hybrids on the surface of avidin-coated particles. This detection format is called affinity-based hybrid collection and was originally described by Syvanen et al. (10). Our use of a biotinylated primer differed, because it allowed direct detection of the PCR product after capture of the product by hybridization to an immobilized capture probe. The assay that we described is simple, sensitive, and more rapid than Southern hybridization (6 h versus 8 h). This assay should permit the rapid and convenient application of PCR technology to the detection of HIV-1 sequences in clinical samples, but further testing of clinical samples will be required to assess the assay's sensitivity and specificity.

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