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Quantitation of Human Immunodeficiency Virus DNA by Using the Polymerase Chain Reaction

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The polymerase chain reaction was used to measure the DNA copy number of human immunodeficiency virus (HIV). Differences in polymerase chain reaction amplification efficiency were controlled by amplifying known amounts of HIV DNA in parallel with samples. This technique is a sensitive, accurate, and reproducible method for the quantitation of HIV DNA.

Human immunodeficiency virus (HIV) causes acquired immune deficiency syndrome (AIDS) (2, 5). Sensitive and quantitative assays that directly measure HIV are urgently needed to monitor the progression of disease and to assess antiviral therapy. The polymerase chain reaction (PCR) assay (11) has been used to specifically amplify HIV DNA sequences (7, 12). The amplified sequences can be increased 10^6-fold or more (14) and specifically detected by nucleic acid hybridization (7, 11, 12). This method is extremely sensitive and capable of detecting very low levels of latent HIV in infected individuals (6, 8).

Quantitation by the PCR assay uses the measurement of the relatively large amount of final product to deduce the amount of target present in the initial sample. The equation relating input and output amounts of DNA following amplification is $Y = S \times (1 + e)^n$, where Y is the amount of DNA after amplification, S is the starting number of target copies, n is the number of cycles performed, and e is the efficiency of the reaction. The value of e can range from zero (no amplification of the target sequence) to one (exact doubling of the target sequence). This variation in efficiency has been a major obstacle in making the PCR assay a quantitative one (1) because the assay is sensitive to reaction conditions and the amount of target material being amplified. It is necessary, therefore, to compare each PCR measurement with a known standard that has similarly been amplified by PCR.

Two approaches to this standardization include the use of internal standards, which involve a eucaryotic gene amplified in the same tube as the target, and external standards, which involve known amounts of the target amplified in different tubes. Internal PCR standards have several disadvantages for the quantitation of HIV DNA, the most serious of which is that when HIV DNA and the internal standard are present in different amounts, they have different amplification efficiencies. In this study we developed a quantitative method to determine HIV DNA copy number with an external standard of known amounts of HIV amplified along with each sample to correct for the difference in the efficiency of amplification based on starting copy number.

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Human subjects. Informed consent was obtained from the patients in this study. Human experimentation guidelines of the U.S. Department of Health and Human Services and those of the University of California were followed in the conduct of the clinical research.

Molecular reagents and virus. pBH10 (courtesy of M. Martin, National Institutes of Health) was grown in *Escherichia coli*, and the plasmid DNA was isolated by established procedures (9). The DNA concentration of the plasmid was determined with a spectrophotometer.

Oligonucleotides SK01, SK02, SK29, SK30, SK38, SK39, SK68, and SK69 (7, 12) were synthesized. Oligonucleotide probes SK03, SK31, SK19, and SK70 were labeled with ^{32}P by using polynucleotide kinase (New England BioLabs, Inc., Beverly, Mass.) (3).

The LAV strain of HIV type 1 (10) was grown in CEM cells, and growth was monitored by reverse transcriptase (13) and antigen production (E. I. du Pont de Nemours & Co., Inc., North Billerica, Mass.).

Isolation of cells and preparation of DNA. Infected and uninfected CEM cells were lysed with 0.5% sodium dodecyl sulfate (SDS), treated with proteinase K (IBI, New Haven, Conn.), phenol extracted, and ethanol precipitated. Nucleic acid was suspended in 1× TE (10 mM Tris chloride, 1 mM EDTA [pH 8.0]), treated with RNase (Sigma Chemical Co., St. Louis, Mo.), phenol extracted, ethanol precipitated, and resuspended in 1× TE. The number of copies of HIV provirus present in a microgram of infected CEM cell DNA was determined by comparison with pBH10 on dot blots with ^{32}P-labeled SK19 and SK70 (12).

DNA was extracted from peripheral blood mononuclear cells (PBMC) of AIDS patients and negative controls by the following protocol. Ten to 15 ml of blood was collected by vein puncture into EDTA-containing tubes. The PBMC were separated on a Lymphoprep gradient, washed once with phosphate-buffered saline, lysed with 400 μl of sterile double-distilled water, and autoclaved for 20 min, followed by a slow exhaust. Samples were centrifuged in an Eppendorf centrifuge at full speed for 2 min, and the supernatants were transferred to new tubes. Samples were equilibrated to 1× TE with 10× TE, treated with RNase for 1 h at 37°C, and treated with proteinase K for 1 h at 55°C. Following phenol extraction and ethanol precipitation, samples were sus-

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pended in 100 μl of double-distilled water and the DNA concentration of the samples was determined with a spectrophotometer.

Amplification protocol. The PCR mixture contained 1× PCR buffer (The Perkin-Elmer Cetus Corp., Norwalk, Conn.); 0.2 mM each dATP, dGTP, dCTP, and dTTP (Pharmacia, Inc., Piscataway, N.J.); 50 pmol of each primer pair (SK01-SK02, SK29-SK30, SK38-SK39, and SK68-SK69); and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus) (4). The final volume of each sample was 50 μl.

One microgram of DNA from each patient and negative control was run for 30 cycles in accordance with the program listed below. Tenfold dilutions ranging from 10^6 to 10^1 copies of HIV provirus from LAV-infected CEM cells were amplified along with the patient samples. All samples were adjusted with lambda DNA (New England BioLabs) to a final DNA concentration of 1 μg per sample.

A DNA thermal cycler (Perkin-Elmer Cetus) was used to cycle the temperature of the samples. Samples were denatured for 3 min at 100°C and quickly cooled. *Taq* polymerase (Perkin-Elmer Cetus) was added, and 50 μl of mineral oil was laid over each sample. Each cycle was as follows: 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. After 30 cycles, samples were incubated at 72°C for 10 min and stored at 4°C until blotted.

Hybridization and construction of standard curves. Dot blots were made with 20 to 25 μl of each amplified sample. Blots were prehybridized at 37 or 50°C in a solution containing 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.01 M EDTA, 5× Denhardt solution, 100 μg of denatured salmon sperm DNA per ml, and 6% SDS. γ-^{32}P-labeled probes were added to the prehybridization mixture to a final concentration of 10^6 cpm/ml, and blots were hybridized overnight at 37 or 50°C. Blots were washed at the hybridization temperature three times; the first wash was done with 2× SSC–0.1% SDS, the second wash was done with 1× SSC–0.1% SDS, and the third wash was done with 0.5× SSC–0.1% SDS. Each wash lasted 30 min.

The blots were exposed to autoradiography film (Eastman Kodak Co., Rochester, N.Y.) for 2 h or overnight at −70°C. Following dot blotting, hybridization, and autoradiography, individual dots were cut out and placed in a scintillation cocktail (Ready Safe; Beckman Instruments, Inc., Fullerton, Calif.). The counts per minute were determined in a scintillation counter set for ^{32}P. Each datum point was determined in duplicate. The counts per minute of samples were always greater than 200.

A standard curve was constructed from the counts obtained from the amplification of a known number of copies of HIV provirus. The counts per minute of patient samples minus the background counts were compared with the curve, permitting calculation of the number of copies of HIV provirus present in the original sample.

Sensitivity and reproducibility of the PCR assay. Amplification for 30 cycles followed by hybridization at 37°C allowed the detection of as few as five copies of pBH10 DNA. Well-to-well differences within the DNA thermal cycler were examined by amplifying samples containing a constant amount of HIV DNA in different wells. Amplification of 42 samples followed by hybridization and scintillation counting showed that the well-to-well differences had a coefficient of variation of 14%. This error also includes the error in the production of the dot blots and the counting of the individual dots. We did not use the 6 positions furthest from the temperature sensors (column 8) on our machine because these positions gave a much higher variation than did the other 42 positions and the use of column 8 increased the coefficient of variation from 14 to 19%.

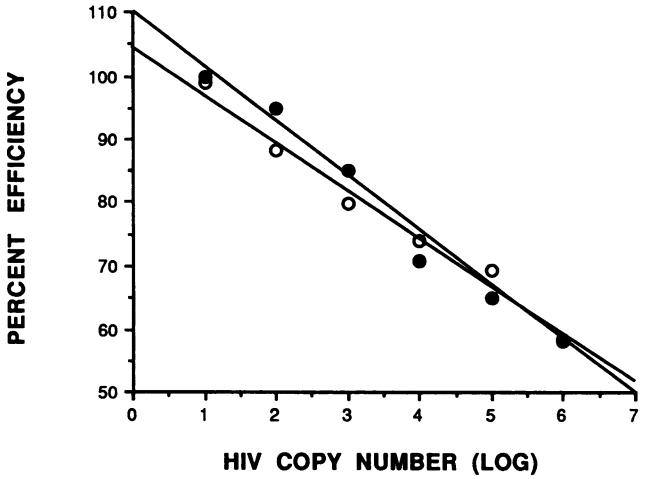

FIG. 1. Comparison of the percent efficiency of amplification versus the logarithm of copy number for primer pairs SK38-SK39 (○) and SK68-SK69 (●). Following amplification of CEM cell DNA with a known copy number for 30 cycles, samples were blotted onto nitrocellulose and the overall efficiency of amplification was determined. Samples amplified with SK38-SK39 were detected with SK19. Samples amplified with SK68-SK69 were detected with SK70.

Determination of PCR efficiency. The efficiency of amplifying HIV DNA with primer pairs SK38-SK39 and SK68-SK69 was determined with both LAV-infected CEM cell DNA and pBH10 DNA. A known number of copies were amplified for 30 cycles in accordance with the above-described protocol. After amplification, 25 μl of each sample was dot blotted along with standards of pBH10 DNA corresponding to the amount of product that would have been generated by amplification efficiencies of 100, 90, 80, 70, 60, and 50%. Following hybridization with γ-^{32}P-labeled SK19 or SK70, individual dots were cut out and counted. Efficiency curves were constructed to determine the efficiency of amplification.

Figure 1 shows the efficiency of amplification with primer pairs SK38-SK39 and SK68-SK69 for 10^1 to 10^6 copies of HIV provirus in 1 μg of total DNA. The efficiency of the reaction decreased with increasing amount of target for both primer pairs. This dependence of the amplification efficiency on the starting amount of target necessitated the use of external standards.

The efficiency of the amplification of HIV provirus was compared with the efficiency of the amplification of plasmid pBH10. Figure 2 shows that the efficiencies of amplifying equal numbers of copies of pBH10 DNA or LAV-infected CEM cell DNA were virtually the same, demonstrating that the results for infected cells can be directly related to copy number.

Accuracy of quantitation by PCR. To determine the accuracy of quantitating the initial copy numbers by PCR, we generated a standard curve by amplification of serial dilutions of pBH10 followed by dot blot hybridization and scintillation counting. Samples of LAV-infected CEM cell DNA, the copy number of which had previously been determined by comparison with pBH10 on Southern blots, were amplified and hybridized along with the pBH10 standards. The counts per minute of the CEM cell samples were compared with the standard curve, and the initial copy

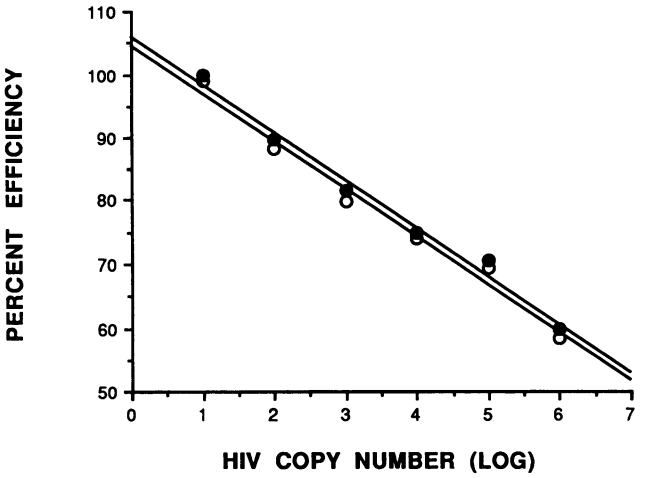

FIG. 2. Comparison of percent efficiency of amplification versus the logarithm of copy number for HIV-infected CEM cell DNA (○) and plasmid (pBH10) DNA (●). Samples containing various concentrations of CEM or pBH10 DNA were amplified for 30 cycles with primer pair SK38-SK39 and blotted onto nitrocellulose so that the efficiency of amplification could be determined.

numbers were calculated. The difference between the initial amount of DNA actually present and the initial amount calculated from the curves did not exceed 13% at any of the concentrations tested. An example of these experiments is shown in Fig. 3.

Determination of HIV copy number in DNA from PBMC of AIDS patients. Figure 4 shows an example of an experiment done to determine the initial number of copies of HIV provirus present in DNA samples from AIDS patient PBMC. Scintillation counting of the individual dots and comparison of the counts per minute in patient samples with the curve derived from the counts per minute of amplified samples with known copy numbers allowed the quantitation of initial

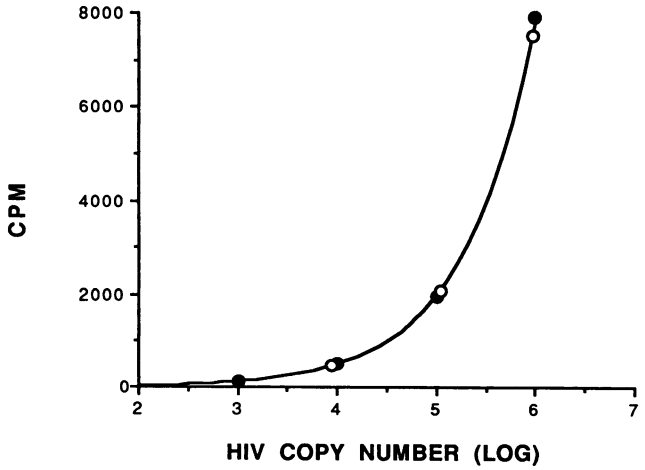

FIG. 3. Plot of counts per minute versus the logarithm of the initial pBH10 DNA copy number following amplification for 30 cycles with SK68-SK69 and dot blot hybridization with SK70. pBH10 DNA (●) was amplified and hybridized, and the counts per minute of each dot were determined by scintillation counting. Samples of HIV-infected CEM cell DNA (○) containing known HIV copy numbers were amplified and hybridized along with the pBH10 standards.

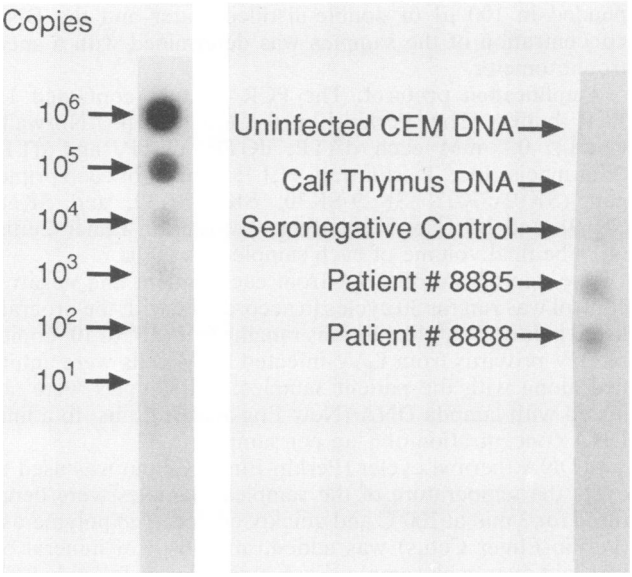

FIG. 4. Determination of the initial copy number of HIV provirus in PBMC DNA from AIDS patients. Samples containing 1 μg of patient PBMC DNA were amplified for 30 cycles with primer pair SK01-SK02 at the same time that controls and standards were assayed. All samples, standards, and controls contained a total of 1 μg of DNA. Samples were blotted onto nitrocellulose and hybridized with SK03 at 50°C, and the blot was exposed to film for 12 h at −70°C. Individual dots were cut out and scintillation counted. By comparison with the standard curve derived from the CEM cell samples, patient 8888 had 56,000 copies of HIV DNA per μg and patient 8885 had 89,000 copies per μg.

copy numbers within the patient samples. Patient 8888 had 56,000 copies of HIV DNA per μg, and patient 8885 had 89,000 copies per μg.

Samples from one patient were quantitated 10 times, and the error based on the mean and standard deviation was calculated. The 14% coefficient of variation found in these samples was consistent with the result of the experiment in which well-to-well differences were examined. We conclude that the use of external standards and PCR amplification is a sensitive, accurate, and reproducible method for the quantitation of HIV.

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