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Quantitation of Human Immunodeficiency Virus Type 1 DNA by Competitive Nested PCR Assay

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SUMMARY A quantitative competitive nested PCR assay was developed for quantifying HIV-1 proviral DNA in clinical samples. A competitor DNA was constructed from a conserved region of the HIV-1 *gag* gene by deleting a sequence of 18 base pairs. We quantitated HIV-1 proviral DNA copy number in clinical samples. Peripheral blood mononuclear cells (PBMCs) from 35 HIV-infected patients with a CD_4 count range of 4-728 cell/mm^3 were analyzed by this method. The copy numbers of HIV-1 DNA detected ranged between 518 to 67,340 copies per 10^6 CD_4^+ T-cells. The copy numbers correlated inversely with the CD_4 counts.

Quantification of HIV-1 DNA and RNA is an essential tool in studies of HIV-1 pathogenesis[1,2] and anti-retroviral drug efficiency.[3] Quantitative competitive polymerase chain reaction (cPCR) has been developed to measure HIV-1 DNA from PBMCs. In the cPCR, the DNA template and the competitor DNA, which serves as an internal control, are coamplified within the same tube and share the same primer recognition sites. Quantification is based on the relative amounts of product derived from the DNA template and the competitor DNA. The PCR products of target template and competitor are differentiated by their different lengths,[4,5] restriction patterns, or specific internal sequence.[5,6] Restriction analysis and gel electrophoresis are used to separate PCR products from templates and competitors that are different in sizes or restriction sites, while temperature gradient gel electrophoresis[7,8] and hybridization with specific internal probes[9] are used to separate those with different internal sequences. However, these available cPCR methods are usually performed by single round PCR and the PCR products are quantified by hybridization to specific oligonucleotide probes in order to achieve sufficient sensitivity.[4-9] This hybridization step increases time and cost of the assay. Currently available competitors that differ in length can be used only in single round PCR, because the deletion sites are located next to primer binding sites. To establish a simple and sensitive quantitative PCR that does not need hybridization, we synthesized a new competitor DNA with internal deletion that can be used in nested PCR. In the present study, we developed a competitive nested PCR using this new competitor DNA to quantitate HIV-DNA in clinical specimens. The competitor with internal 18 base pair deletion was generated by PCR mutagenesis using internal primers and then extended to the whole length by overlapping spliced method. In order to verify the accuracy of this newly developed assay, we measured the HIV-1 DNA level in cell line 8E5 with a known copy number of HIV-1 DNA.[10] We also used this assay to measure HIV-1 DNA loads in clinical specimens as a preliminary

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evaluation of this assay. The competitor has been cloned into a plasmid vector downstream to T7 RNA polymerase promoter. This construct can be used to produce RNA competitors for further development of a competitive RT-PCR for HIV-1 RNA viral load measurements.

MATERIALS AND METHODS

Subjects and cell preparation

EDTA blood samples from 35 HIV infected patients from the HIV-Clinic at Siriraj Hospital were collected. None of the patients had received any anti-retroviral drug. The CD_4 counts ranged from 4-728 cells/mm^3. PBMCs were isolated by centrifugation on Ficoll-Hypaque density gradient. DNA was extracted by QIAGEN blood kit and DNA equivalent to 1.5 x 10^5 PBMCs per sample was amplified in a PCR reaction. DNA was extracted from the PBMCs of seronegative individuals was used as negative control in this assay.

Synthesis of the competitor DNA

For the competitive analysis, we used primers SK380/390 and SK38/39 to amplify the HIV-1 *gag* gene. This primer set has been shown to perform well with high specificity and sensitivity for subtype E viruses in Thailand.[11] The competitor was generated by PCR mutagenesis and an overlap extension method. Plasmid pHXB2 was used as template for PCR to generate the competitor. First, pSKAN was synthesized as described previously.[4] Briefly, pHXB2 was amplified with the SK38Δ - SK39 primer pair. The SK38Δ carried a 18 base deletion from position 1579 to 1596, as shown in Fig. 1 and Table 1. Subsequently, this product was reamplified with the SK38 - SK39 primer pair in order to add the 5' end of SK38 primer site. Second, a K1 fragment with 18 base deletion was generated using purified pSKAN and SK390 as primers. PCR reaction solution containing pHXB2 as template, pSKAN and SK390 as primers, standard PCR buffer (10 mM Tris-HCl, 50 mM KCl and 0.1% Triton®X-100) and 1.5 mM $MgCl_2$ was denatured at 100°C for 10 minutes, then chilled on ice. Subsequently, 200 μM of each dNTPs and 2.5 U *Taq* DNA polymerase were added.[12] Amplification was performed with 35 repeated cycles consisting of 95°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute and additional cycle at 72°C for 10 minutes. Finally, the competitor was produced in another PCR reaction with pHXB2 as template, SK380 and purified K1 as primers. Amplification was done under the same conditions as described for K1 synthesis. A large amount of the DNA competitor was purified and quantitated by using UV spectrophotometer and visual intensity of ethidium bromide fluorescence of DNA by Saran wrap method.[13] The copy number of competitor was then calculated.

Competitive nested-PCR

Competitive nested-PCR was done in a total volume of 50 μl, containing 1 x standard PCR buffer (10 mM Tris-HCl, 50 mM KCl and 0.1% Triton®X-100), 1.5 mM $MgCl_2$, 200 μM(each) dNTPs, 20 pmol each of *gag* primer SK380-SK390 and 2.5 U of *Taq* DNA polymerase, a variable copy number of DNA competitor (5, 25, 50 and 100) and finally, DNA equivalent to 1.5 x 10^5 PBMCs were added into each PCR mixture. PCR reactions were denatured at 95°C for 5 minutes followed by 35 repeated cycles consisting of 95°C for 1 minute, 60°C for 1 minute, and 72°C

Table 1 Oligonucleotide primers for competitor construction and nested PCR

Primer	No. of base (position)	Sequence
SK38Δ	44 (1559-1620)	5'-CCTATCCCAGTAGGAGAAATCCTGGGATTAAATAAAATAGTAAG-3'
SK38	28 (1551-1578)	5'-ATAATCCACCTATCCCAGTAGGAGAAAT-3'
SK39	28 (1665-1638)	5'-TTTGGTCCTTGTCTTATGTCCAGAATGC-3'
SK380	24 (1236-1250)	5'-CAGGGACAAATGGTACATCAGGCT-3'
SK390	29 (1695-1717)	5'-GGGAAATCTCTGATACATCTGCCCAAGT-3'

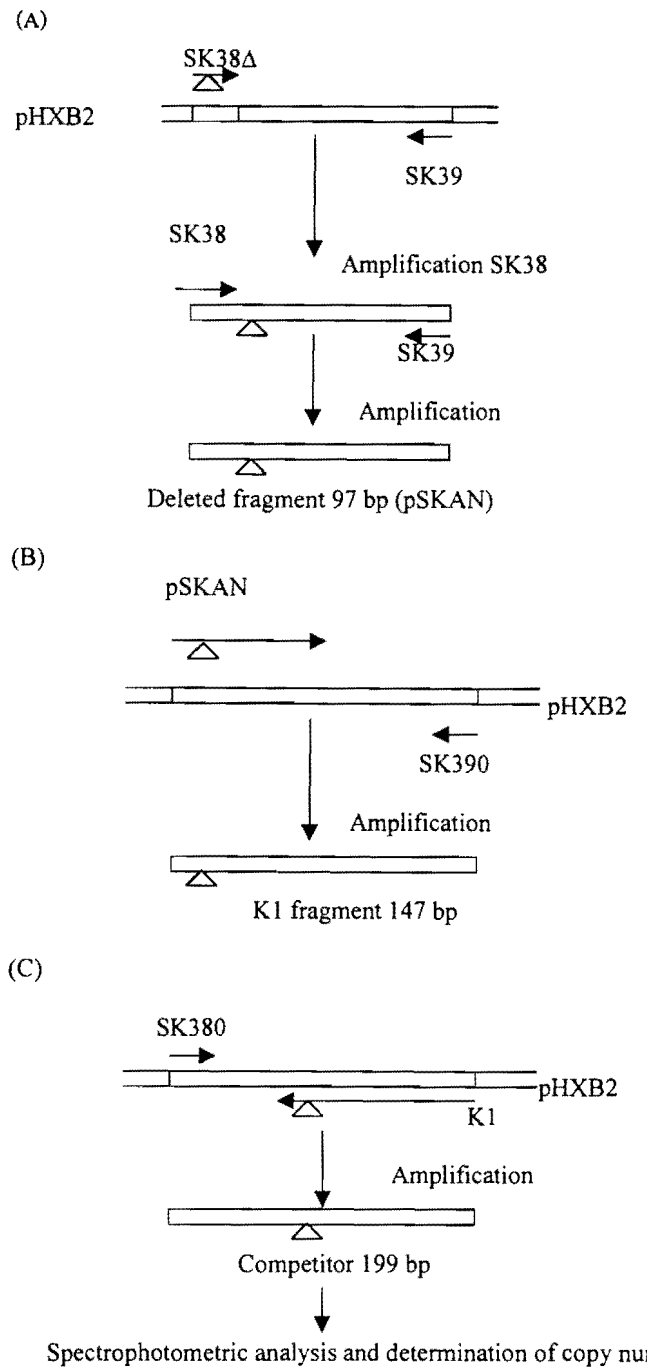

Fig. 1 Schematic diagram representation of the construction of a competitor DNA with internal 18 bp deletion. Plasmid pHXB2 was used as template for PCR to generate the competitor. PCR primers are listed in table 1. For generation of pSKAN, pHXB2 was amplified with SK38Δ, carrying a 18 base deletion (tringle) and SK39 (A). Subsequently, the first-round PCR product was re-amplified with a SK38-SK39 primer pair. Second, a K1 fragment with 18 base deletion was generated using purified pSKAN and SK390 as primers (B). Finally, the competitor was made using SK380 and purified K1 as primers (C).

for 1 minute and an additional extension at 72°C for 10 minutes. The PCR product from primary PCR was reamplified with inner primer pair SK38-SK39. The reaction step was performed under the same condition as primary PCR except that primer annealing was done at 55°C for 1 minute. Proviral DNA was calculated as described previously.[4] Briefly, the amplification product was run on 10% polyacrylamide gel at 150 V for 90 minutes in order to separate the 115 bp wild-type DNA from the 97 bp competitor DNA product. After gel electrophoresis and ethidium bromide staining, the relative intensity in each lane of both DNA was measured by digital photograph and the peak areas (WA, wild-type area; DA, competitor area) of both amplified products were calculated by Gel-Pro analysis computer software. DA was corrected (DAc) for its lower molar ethidium bromide incorporation as follows: DAc = DA x (wild-type length/ competitor length) = DA x 1.185. The DAc/WA ratio was calculated for each lane and plotted against the copy number of the competitor. Finally, the wild-type DNA copy was extrapolated from the standard curve at ratio = 1.

RESULTS

To determine the sensitivity of competitive nested PCR method by using end-point dilution amplification, the stock of DNA competitor was 10-fold serially diluted from 10^{-1} to 10^{-10} and 1 µl of each dilution was amplified in duplicate with HIV-1 *gag* specific primers. The increase of the amplification product was linear between 3 and 3 x 10^4 copies. The limit of detection was 3 copies per reaction. This level of sensitivity was higher compared to 10 copies that were reported

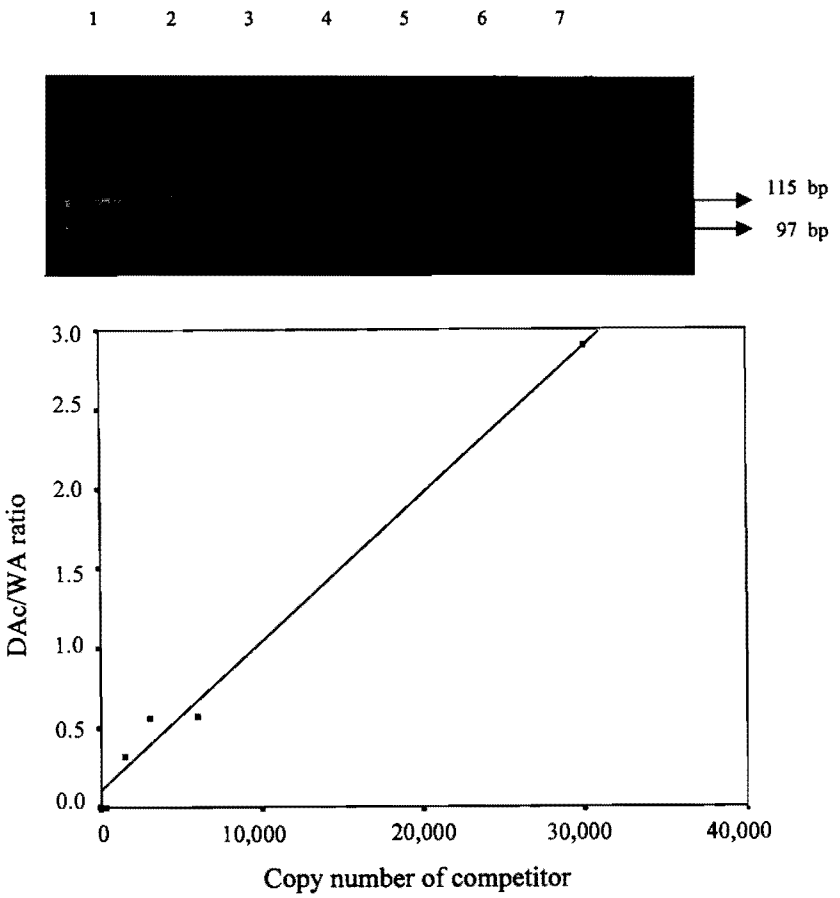

Fig. 2 Optimization of competitor DNA. (A) Co-amplification of 1 x 10^4 8E5 copies (upper band, 115 bp) and the competitor (lower band, 97 bp). Lane 1, marker; lanes 2 to 6: 300, 1,500, 3,000, 6,000, 30,000 copies of competitor; lane 7, reagent blank, respectively. (B) The linear regression curve plot between DAc/WA ratio and the copy number of competitor. The amount of DNA calculated by this curve was equivalent to 9,550 copies.

using single round PCR and hybridization on the competitor pSKAN.[4] To verify the accuracy of our competitive nested PCR, 8E5 cells that contain one copy of HIV-1 DNA per cell were used to coamplify with competitor. Ten thousand copies of 8E5 were subdivided into five parallel reactions and each reaction was coamplified with an increasing copy number of DNA competitor (300, 1,500, 3,000, 6,000, and 30,000). The 8E5 products were of 115 bp in length, while the competitor products were of 97 bp. As shown in Fig. 2, the copy number of 8E5 calculated from a linear regression curve was equivalent to 9,950 copies. Next, we examined the HIV-1 DNA in clinical samples from 35 HIV-infected subjects. In this study, purified DNA equivalent to 1.5 x 10^5 PBMCs was coamplified with 5, 25, 50, and 100 copies of competitor DNA. The raw data of HIV-1 DNA copies per 1.5 x 10^5 PBMCs were normalized to HIV-1 DNA copies per million CD_4^+ T cells by using the percentages of CD_4^+ T cells of the same sample. None of the extracted DNA from HIV-1 seronegative individuals was positive by this PCR assay. As shown in Fig. 3, the number of HIV-1 DNA copies ranged from 518 to 67,340 per million CD_4^+ T cells and the levels of HIV-1 DNA were inversely correlated with the CD_4^+ count ($P < 0.01$; Spearman rank coefficients of correlation, -0.854). To check the run-to-run variability of this competitive nested PCR method, HIV-1 DNA was quantified repetitively from PBMCs of two patients. Each sample was analyzed three times. The amounts of HIV-DNA by the three analysis were 294, 287, and 302 copy/10^6 CD_4^+ T cells for the first sample and 103, 116, and 101 copy/10^6 CD_4^+ T cells for the second sample.

DISCUSSION

Quantification of HIV-1 DNA or RNA is important for the studies of HIV pathogenesis, monitoring of disease progression and response to anti-retroviral therapy. Various techniques have been established for detection of nucleic acids including PCR technique. At present, competitive PCR is the most reliable method for quantitation of HIV DNA in clinical samples. Coamplification of competitor provides an internal control for amplification efficiency. However, the available competitor DNA is currently designed to be used in a single-round PCR only, which needs subsequent hybridization to obtain sufficient sensitivity. We described here a competitive nested PCR assay for quantitation of HIV-1 *gag* region. A new competitor DNA was constructed and optimized for competitive nested PCR with high sensitivity and did not need hybridization. We chose the primer pairs SK380-SK390 and SK38-SK39 in our competitive nested PCR because this primer set

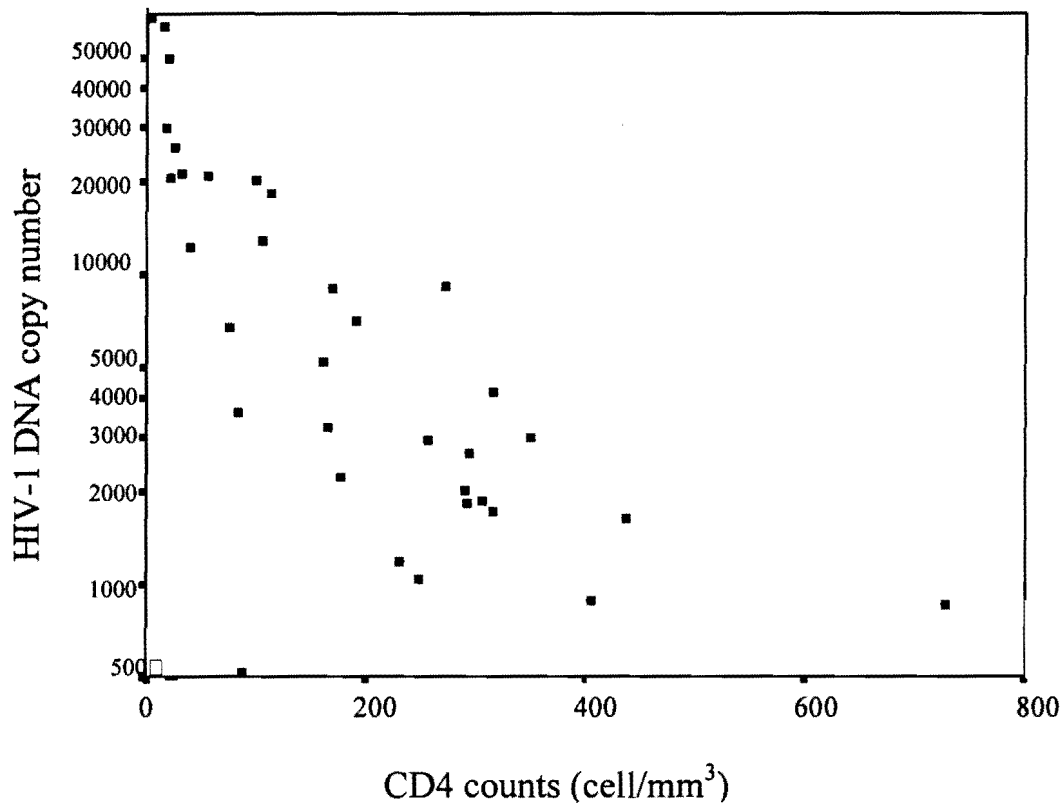

Fig. 3 Scatter plots show the correlation between CD_4 count (X-axis) and copy number of proviral DNA determined by the competitive nested PCR.

is suitable for the *gag* gene of HIV-1 subtype E in Thailand and it has been used routinely for qualitative detection of HIV-1 DNA in our laboratory. Our PCR system could detect at least 3 copies of HIV-1 DNA. We have validated the accuracy of our competitor PCR assay by using known HIV-1 DNA copy number. In a preliminary evaluation of the quantitative PCR, we demonstrated the inverse correlation between HIV-1 DNA and CD_4 counts. This supported the accuracy of the assay and its role in monitoring disease progression.

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