

New estimation method for highly sensitive quantitation of human immunodeficiency virus type 1 DNA and its application

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Abstract

A new estimation method for quantitation of HIV-1 DNA was established by introducing a pre-quantitation polymerase chain reaction (PCR) before conventional real-time PCR. Two alternative methods for estimating the copy number can be used: the first method utilizes the rate of $\beta 2$ -microglobulin ($\beta 2M$) gene amplification during the pre-quantitation PCR, and the second utilizes a calibration curve of the crossing point of real-time PCR versus the standard HIV-1-plasmid concentration. These methods could be used to reproducibly and accurately detect a provirus density down to five copies/ 10^6 cells (for methods 1 and 2, inter-assay CV = 17 and 16% and accuracy = 81 and 92%, respectively). The levels of HIV-1 DNA could be measurable using as little as 100 μ l of whole blood or buffy coat cells. Using a combination of a conventional and highly sensitive methods, we found that the amount of HIV-1 DNA ranged from 2 to 5960 copies/ 10^6 cells (median of 830 copies/ 10^6 cells) in CD4-positive T lymphocytes isolated from 30 patients responding well to highly active antiretroviral therapy (HAART). Thus, the highly sensitive method developed in this study allows estimation of the HIV-1 reservoirs in peripheral CD4-positive T lymphocytes of patients responding well to HAART.

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1. Introduction

Due to the recent development of highly active antiretroviral therapy (HAART), it has been possible to effectively suppress the progression of HIV-1 infectious disease to AIDS.

Abbreviations: HIV-1, human immunodeficiency virus type 1; HAART, highly active antiretroviral therapy; PCR, polymerase chain reaction; $\beta 2M$, $\beta 2$ -microglobulin; WBC, white blood cells; Cp, crossing point; CV, coefficient of variation; VL, viral load

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In many cases, the treatment of HIV-1-infected patients with HAART, which uses a combination of reverse transcriptase and protease inhibitors, can rapidly suppress plasma HIV-1 RNA levels below the detectable level (Mellors et al., 1996; Perelson et al., 1997). Therefore, the effectiveness of the treatment has been monitored by measuring plasma viral load.

Given their mode of action, it is clear that reverse transcriptase and protease inhibitors do not eliminate the HIV-1 infected cells. Consequently, quantitation of HIV-1 DNA in virus-infected cells has become important for estimating the residual viral reservoir in patients receiving HAART (Chun

et al., 1997; Désiré et al., 2001; Ibanez et al., 1999; Izopet et al., 1998; Riva et al., 2001, 2003; Sharkey et al., 2000; Yerly et al., 2000). A recently developed technique, real-time polymerase chain reaction (PCR), has made this possible because it can rapidly and specifically quantify a target DNA without complex manipulations (Damond et al., 2001; Désiré et al., 2001; Izopet et al., 1998; Riva et al., 2001; Wada et al., 2004; Walker, 2002; Zhao et al., 2002).

Recently, we demonstrated the clinical significance of HIV-1 DNA quantitation by real-time PCR as measured with the LightCycler system with a detection limit of 500 copies/ 10^6 cells (Wada et al., 2004). However, the HIV-1 DNA levels in 30% of therapy-naïve or HAART-receiving patients could not be quantified with the conventional method because of a relatively high detection limit. Therefore, a more sensitive method must be developed to quantify much lower levels of HIV-1 DNA, which is particularly important for following patients responding well to HAART.

2. Materials and methods

2.1. Quantitation of HIV-DNA

The two methods established in this study for quantifying HIV-1 DNA are shown in Figs. 1 and 2. Method 1 was based on an estimation in which the HIV-1 DNA copy numbers were calculated by dividing the amount of amplified HIV-1 DNA by the rate of human β 2-microglobulin (β 2M) gene amplification (Fig. 1). First, both HIV-1 DNA and β 2M genes were simultaneously amplified in the same tube. Second, the copy numbers of amplified HIV-1 DNA was separately quantified by real-time PCR. A standard curve was then generated by plotting the crossing point (Cp) from real-time PCR as a function of the concentration (copy number) of HIV-1 standard plasmid (serial 10-fold dilutions from 10^{10} to 10 copies). The Cp is the PCR cycle number where the amount of PCR product is the same in all curves (Rasmussen, 2001). As shown

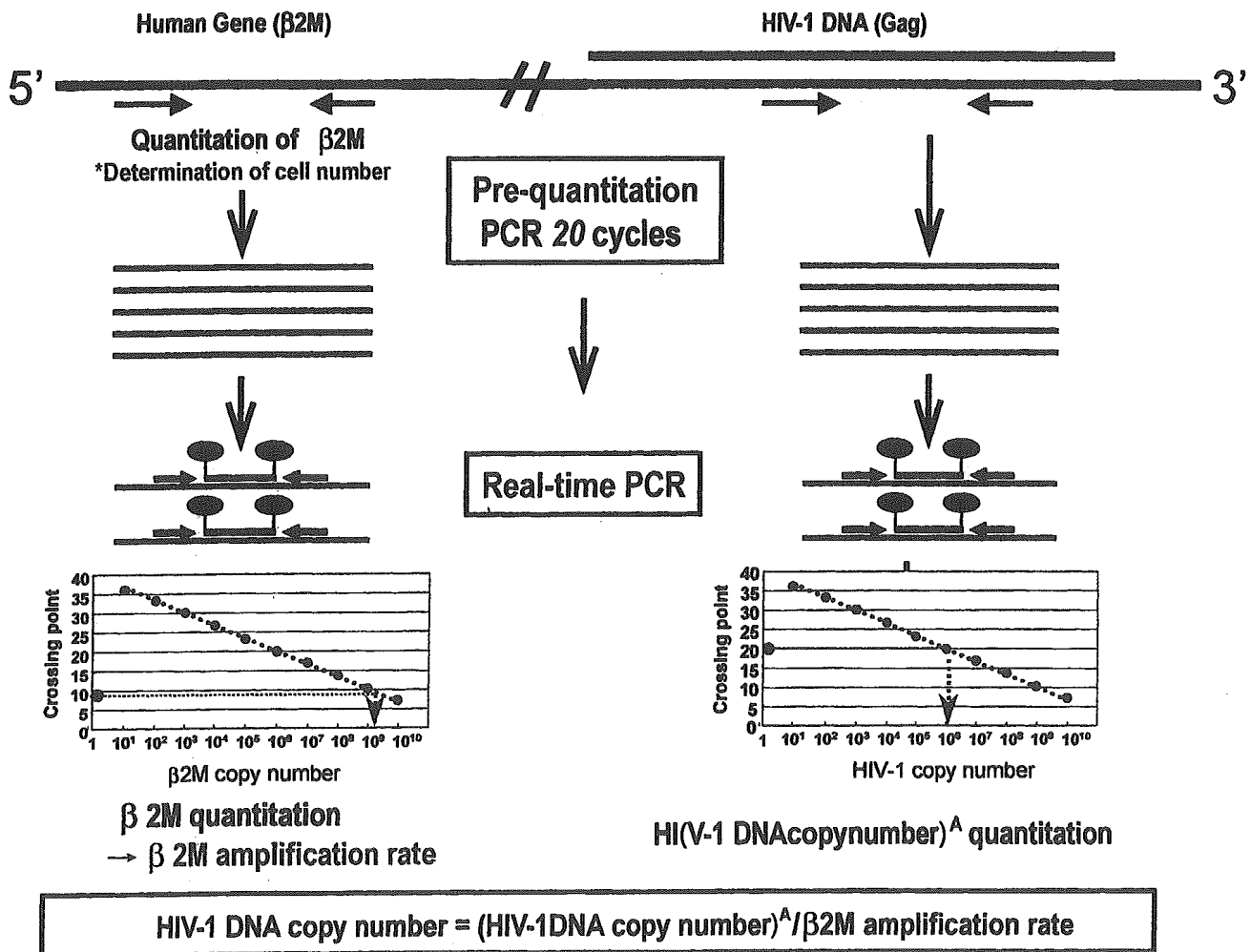


Fig. 1. Method 1: first, both HIV-1 DNA and β 2M genes are simultaneously amplified in one tube (pre-quantitation PCR). Second, the copy numbers of amplified HIV-1 DNA, $(\text{HIV-1 DNA copy numbers})^A$ and β 2M genes are separately quantified by real-time PCR. The amplification rate of β 2M genes are determined by dividing the PCR-amplified copy numbers by the original copy numbers before pre-quantitation PCR. Finally, HIV-1 copy numbers are calculated as amplified HIV-1 DNA copy numbers/ β 2M gene amplification rate. Using the standard curve (blue dotted line), the amount of HIV-1 (red arrow head) is estimated from the measured Cp (red circle).

in Fig. 1, by using the standard curve (blue dotted line), the amount of HIV-1 (red arrow head) was estimated from the measured C_p (red circle). Next, the amplification rate of β 2M genes was determined by dividing the pre-quantitation PCR-amplified copy numbers by the copy numbers obtained before the PCR. Finally, the HIV-1 copy number was calculated as the amplified HIV-1 DNA copy number divided by the β 2M gene amplification rate.

In method 2, HIV-1 copy numbers were calculated according to a standard curve obtained from the amplified HIV-1 standard plasmids (Fig. 2). First, pre-quantitation PCR was performed as described for Method 1. HIV-1 DNA samples and serially diluted pUC-III B standard plasmids (1000, 100, 50, 10, or 5 copies/assay) were amplified at the same time in different tubes. Second, the C_p of each sample was determined by real-time PCR using aliquots of amplified HIV-1 DNA and pUC-III B standard plasmids. The quantity of HIV-1 DNA was determined using a standard curve of the C_p obtained in real-time PCR versus the logarithm of the standard plasmid concentration in pre-quantitation PCR. Finally,

the copy numbers of HIV-1 DNA were determined using the C_p of the HIV-1 sample obtained in real-time PCR.

Intra- and inter-day precision values for the methods were estimated by quantifying four different copy numbers of HIV-1 five times on the same day and on three separate days, respectively, to obtain coefficient of variation (CV), which is the standard deviation divided by the average.

Accuracy was determined as the percentage of the nominal HIV-1 copy number.

2.1.1. Preparation of pre-quantitation PCR primers

To obtain the highly conserved nucleotide sequences for creation of pre-quantitation PCR primers, we determined the DNA sequences of the HIV-1 gag p17 gene flanking the conventional real-time PCR primers. DNA was amplified by nested-PCR with Pyrobest DNA polymerase (TaKaRa, Shiga, Japan). External primers, ExF (5'-tacataatcacagtagcagtcctctattgtgtgca-3') and ExR (5'-tctaaagggctctttgttct-3'), and the internal primers, InF (5'-agtcctctattgtgtgcatcaaagga-3') and InR

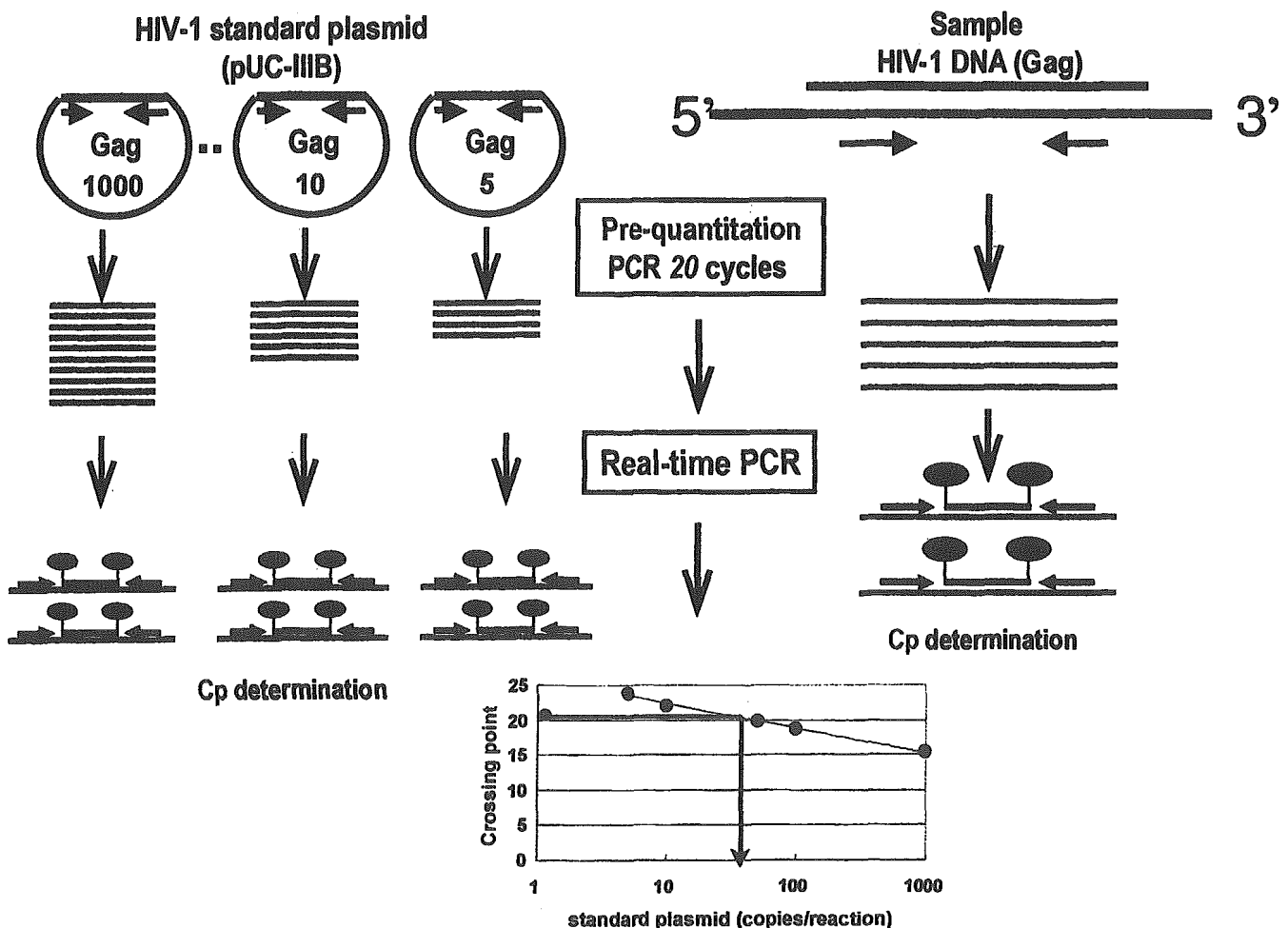


Fig. 2. Method 2: first, pre-quantitation PCR is performed as is performed in method 1. HIV-1 DNA samples and serially diluted pUC-III B standard plasmid (1000, 100, 50, 10, or 5 copies/assay) are separately amplified at the same time in different tubes. Second, the C_p of each sample is determined by real-time PCR where aliquots of the amplified HIV-1 DNA and pUC-III B standard plasmid are used. Quantitation is based on a standard curve of the C_p obtained in real-time PCR vs. the log standard plasmid concentrations in pre-quantitation PCR. Finally, the copy numbers of HIV-1 DNA are determined using the C_p of the HIV-1 sample obtained in real-time PCR.

(5'-tgtcttatgtccagaatgct-3'), were used for the nested-PCR. PCR products of 615 bp were labeled with an ABI Prism dye terminator sequencing kit and were directly sequenced using an ABI Prism 310 genetic analyzer (Applied Biosystems, Tokyo, Japan).

The nucleotide sequences of subtype B HIV-1 from eight patients undergoing HAART were determined and deposited in DDBJ Genbank under accession numbers AB112051 to AB112058. The forward primer GF4 (5'-atggatcatcagccatcaccta-3') and the reverse primer GR4 (5'-tgctatgtcacttccccttggt-3') for pre-quantitation PCR were designed to have a melting temperature of approximately 50 °C in the highly conserved region.

2.1.2. Nested real-time PCR

The pre-quantitation PCR mixture (200 µl total volume) contained 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 200 nM of each 4dNTP, 500 nM each of GF4 and GR4 primers, 2.5 Units of Taq DNA polymerase (Roche Diagnostics, Tokyo, Japan), and 50 µl of purified total cellular DNA. To perform a precise quantitation, it is essential to use the total volume of DNA extract rather than aliquot (see Section 2.1.4). The thermal cycler (MiniCycler; MJ Japan, Tokyo, Japan) was programmed to perform denaturation at 94 °C for 1 min, followed by 20 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 70 °C for 1 min.

Conventional real-time PCR was successively performed using a LC fast start DNA master mix hybridization probe kit (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. The conditions were as follows: denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 10 s and annealing and extension at 60 °C for 30 s. Each PCR mixture (20 µl total volume) contained 2 µl of 10× DNA master mix, 4 mM MgCl₂, 500 nM each of HIV-1 Gag1 and Gag2 primers, and 400 nM TaqMan probe. Amplification, data acquisition, and analysis were performed using the LightCycler system. The specificities of Gag1, Gag2, and the TaqMan probe (Gag86T) were fully described in our previous report (Wada et al., 2004).

2.1.3. Preparation of the standard plasmid

The HIV-1 standard plasmid (pUC-III_B) was constructed by inserting HIV-1 III_B DNA without LTR into the pUC-118 vector (TaKaRa, Shiga, Japan). The β2M standard plasmid (pGEM-β2M) was constructed by inserting the β2M exon2 whole sequence into the pGEM-T vector (Invitrogen, Tokyo, Japan). The concentration of these standard plasmids was determined by absorbance at 260 nm.

2.1.4. DNA extraction and determination of cell number

DNA extraction was performed using the QIAamp DNA blood mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. DNA was recovered in 50 µl of

diethylpyrocarbonate-treated water. The DNA concentration was determined by absorbance at 260 nm. Cell numbers in the assayed samples were calculated by determining the β2M copy numbers based on the fact that one cell contains two copies of the β2M gene and that there are no pseudogenes (Beillard et al., 2002). In addition, we verified this point by comparing the DNA concentration to the β2M copy numbers assuming that one cell contains 6 pg DNA. Quantitation of β2M was carried out under the same conditions as HIV-1 quantitation and using the forward primer β2M-F2 (5'-cagcaaggactggctttctatct-3'), the reverse primer β2M-R (5'-accaccactaactatcttg-3'), and a TaqMan β2M probe (5'-FAM-cactgaaaagatgagatgcctgccgtg-TAMRA-3'). The primers and TaqMan probe were designed to correspond to exon2 of the β2M gene.

2.2. Cell lines

MOLT4 and ACH2 cells were used as negative and positive controls, respectively. ACH2 cells are known to possess one HIV-1 provirus per cell (Folks et al., 1989). ACH2 and MOLT4 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum in a 5% CO₂ incubator at 37 °C.

2.3. Patients

Thirty patients receiving HAART were enrolled in this study. HIV-1 DNA was quantified after obtaining informed consent.

2.3.1. Quantitation of plasma HIV-1 RNA

Plasma HIV-1 RNA load was measured by an Amplicor HIV-1 Monitor, version 1.5 (Roche Diagnostics Systems, Tokyo, Japan).

2.3.2. Purification of CD4-positive T lymphocytes

CD4-positive T lymphocytes were purified from whole blood by StemSep column chromatography (Stem Cell technologies, Vancouver, BC, Canada). Collected cells were washed and then resuspended in phosphate-buffered saline (pH 7.4). The CD4-positive T lymphocytes were more than 98% pure as estimated by flow-cytometry, and 1–5 × 10⁵ cells were usually used for subsequent DNA purification.

3. Results

3.1. Determination of the optimum cycle number for pre-quantitation PCR

Pre-quantitation PCR should be performed under conditions where there is a linear relationship between the amount of PCR product and the number of PCR cycles because the

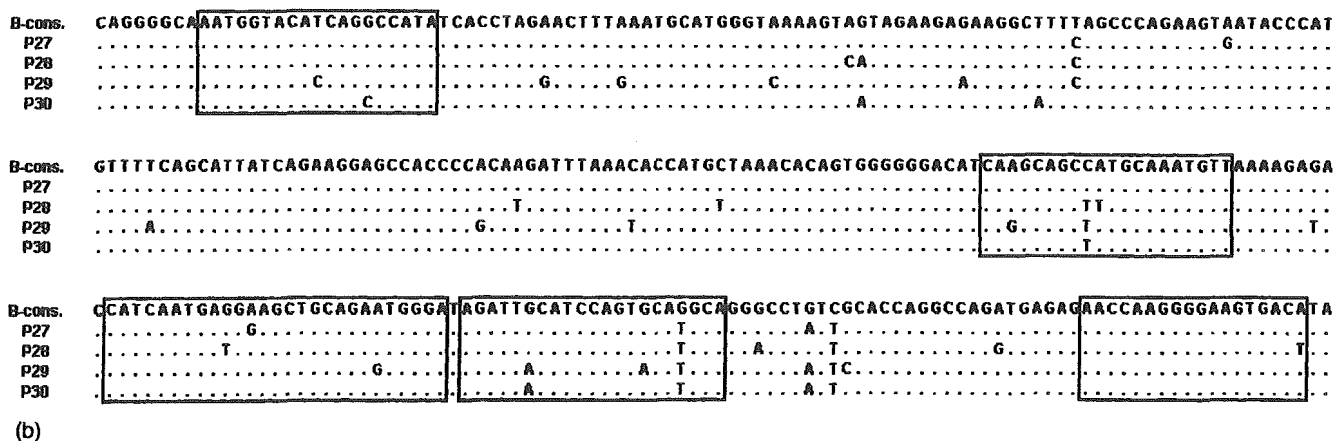
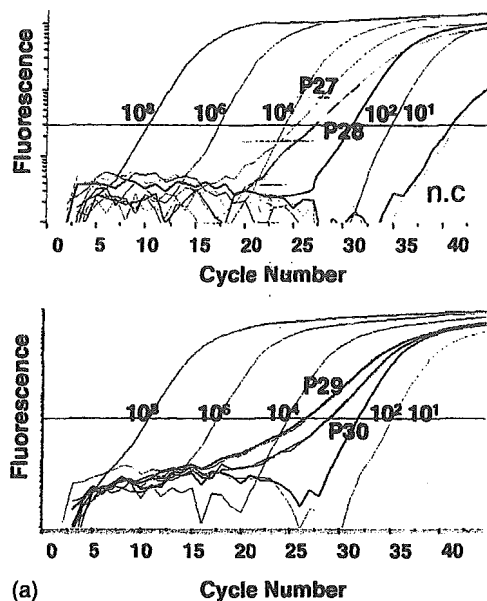


Fig. 3. Analysis of ultra-low levels of HIV-1 DNA in samples P27, P28, P29, and P30. (a) Fluorograms of real-time PCR in these samples. (b) Nucleotide sequences including the region flanking the primer sites used in the pre-quantitation PCR. B-cons, base sequence of HXB2 used as a mother sequence.

amplification rate determined in this step is used to the calculate HIV-1 copy number. We found that the generation of products increased linearly for up to 30 cycles of PCR when 10^5 – 10^4 equivalent copies of ACH2 HIV-1 DNA were used. When we used 10^6 copies, the generation of products increased linearly for up to 20 cycles, but, thereafter, there was a decreased rate of PCR product synthesis (data not shown). For this reason, we adopted 20 cycles for the pre-quantitation PCR.

3.1.1. Validation of method 1

To validate our method for low HIV-1 DNA concentrations, we prepared authentic samples by mixing a quantity of ACH2 DNA equivalent to 100, 50, 10, or 5 cells with an amount of MOLT4 DNA equivalent to 10^6 cells (Table 1). Cell numbers were estimated from the $\beta 2M$ copy number and the assumption that two $\beta 2M$ genes correspond to one cell. For samples includ-

ing 100, 50, 10, and 5 copies of HIV-1 DNA, the average inter-assay copy numbers (average \pm S.D.) were 98 ± 13 (CV = 13.0%), 44 ± 6 (CV = 12.8%), 9 ± 0.7 (CV = 7.8%), and 4 ± 0.4 (CV = 11.4%), respectively (Table 1). The accuracies (%) of the corresponding experiments were 97.7 ± 12.7 , 88.3 ± 11.3 , 87.2 ± 6.8 , and $72.4 \pm 8.3\%$, respectively. Similar results were obtained comparing the inter-assay variability (Table 1).

3.1.2. Validation of method 2

For samples including 100, 50, 10, or 5 copies of HIV-1 DNA, the average intra-assay copy numbers (average \pm S.D.) were 99 ± 5 (CV = 5.3%), 48 ± 3 (CV = 6.6%), 9 ± 1.2 (CV = 12.9%), and 5 ± 0.5 copies (CV = 11.4%), respectively. The accuracies (%) of the corresponding experiments were 98.7 ± 5.2 , 95.7 ± 6.3 , 92.3 ± 11.9 , and $93.9 \pm 10.7\%$, respectively. Method 2 has the benefit that determination of the amplification rate is not necessary.

Table 1
The validation of the highly sensitive real-time PCR method

	HIV-1 DNA					
	Intra-assay (<i>n</i> = 5)			Inter-assay (<i>n</i> = 15)		
	Average ± S.D.	CV (%)	Accuracy (%)	Average ± S.D.	CV (%)	Accuracy (%)
Method 1 ^a ACH2 (cells/assay)						
100	98 ± 12.7	13.0	97.7 ± 12.7	97 ± 12.9	13.2	97.4 ± 12.9
50	44 ± 5.7	12.8	88.3 ± 11.3	46 ± 6.3	13.7	91.7 ± 12.6
10	9 ± 0.7	7.8	87.2 ± 6.8	9 ± 1.0	11.0	86.6 ± 9.5
5	4 ± 0.4	11.4	72.4 ± 8.3	4 ± 0.7	16.9	80.7 ± 13.6
Method 2 ^b ACH2 (cells/assay)						
100	99 ± 5	5.3	98.7 ± 5.2	96 ± 6.5	6.8	96.3 ± 6.5
50	48 ± 3	6.6	95.7 ± 6.3	47 ± 3.6	7.6	94.8 ± 7.2
10	9 ± 1.2	12.9	92.3 ± 11.9	9 ± 1.3	14.0	90.3 ± 12.6
5	5 ± 0.5	11.4	93.9 ± 10.7	5 ± 0.7	16.0	91.5 ± 14.7

^a Gene amplification rate during pre-quantitation PCR.

^b The standard curve between HIV-1 standard plasmid and PCR crossing points was used to calculate the HIV-1 DNA copy numbers as described in Section 2.

Table 2
Application 1: amounts of HIV-1 DNA in CD4-positive T lymphocytes from patients responding well to HAART

No.	HIV-1 DNA copies/10 ⁶ CD4		Sex	CD4 (cells/μl)	Months ^c	Months ^d
	Conventional ^a	HS ^b				
1	5960		M	333	43	26
2	3400		M	219	17	7
3	2950		M	280	63	12
4	1510		M	838	17	1
5	1490		M	956	45	6
6	1350		M	1142	21	15
7	1230		M	219	26	23
8	1210		M	280	63	12
9	960		F	283	63	1
10	940		M	602	37	12
11	920		M	383	22	1
12	890		M	1273	48	27
13	830		M	857	26	24
14	770		M	496	27	25
15	750		M	369	10	6
16	640		M	596	39	28
17	630		M	891	47	20
18	560		F	373	43	43
19	<dl ^e	487	M	354	14	7
20	<dl	412	M	171	3	2
21	<dl	224	M	570	51	42
22	<dl	109	M	547	10	3
23	<dl	46	M	847	24	24
24	<dl	28	M	260	51	42
25	<dl	28	F	233	63	1
26	<dl	7	M	505	48	4
27	<dl	4	M	216	7	3
28	<dl	3	M	1260	76	43
29	<dl	3	M	1090	61	27
30	<dl	2	M	398	27	10

^a Conventional real-time PCR.

^b Highly sensitive real-time PCR.

^c Duration after the first examination.

^d Duration after VL was suppressed below the detection limit.

^e Less than detection limit (500 copies/10⁶ cells).

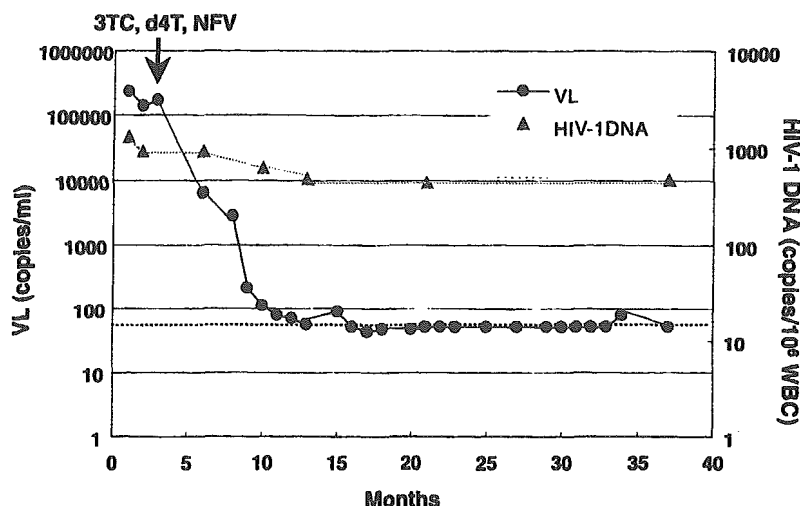


Fig. 4. Changes in the HIV-1 DNA levels in WBC and the plasma viral load after the onset of HAART. Nucleoside reverse transcriptase inhibitors (3TC and d4T) and the protease inhibitor (NFV) were administered for HAART. The red dotted line represents the detection limit (50 copies/ml) of HIV-1 RNA.

3.1.3. Application 1: determination of the amount of HIV-1 DNA in CD4-positive T lymphocytes from patients responding well to HAART

Table 2 shows the amounts of HIV-1 DNA in CD4-positive T lymphocytes determined for 30 patients responding well to HAART. Method 1 was used to estimate HIV-1 DNA copy numbers. The amounts of HIV-1 DNA in 18 of 30 patients (60%) could be estimated by conventional real-time PCR, and they varied from 560 to 5960 copies/ 10^6 cells. Because the HIV-1 DNA copy numbers of the remaining 12 patients were under the detection level of the conventional real-time PCR (<500 copies/ 10^6 cells), they were subsequently measured using our highly sensitive assay. The HIV-1 DNA copy number from all patients could be quantified, and they ranged from 2 to 487 copies/ 10^6 cells. We were concerned that the copy numbers found in patients 27, 28, 29, and 30 were artificially low, although the fluorograms obtained from real-time PCR showed good sigmoidal curves (Fig. 3a). To eliminate the possibility that mismatches of primers or TaqMan probe occurred in these cases, the HIV-1 DNA region used for real-time PCR was amplified and sequenced. As shown in Fig. 3b,

there were no significant mutations that would interfere with real-time PCR, suggesting that the results of the real-time PCR were not artificial. Finally, all 30 cases were positive for HIV-1 DNA. The amount of HIV-1 DNA could be quantified in all cases by using highly sensitive real-time PCR, and they ranged from 2 to as many as 5960 copies/ 10^6 cells with a median of 830 copies/ 10^6 cells. There was no correlation between HIV-1 DNA levels and CD4-positive T lymphocyte counts, duration after the first examination, or duration after viral load (VL) suppression (Table 2).

3.1.4. Application 2: HIV-1 DNA levels in whole blood samples

HIV-1 DNA was quantified using two different volumes of whole blood from five patients receiving HAART. Although HIV-1 DNA could not be quantified by conventional real-time PCR, HIV-1 DNA was successfully quantified in 100 μ l of whole blood using our highly sensitive method, even in a sample containing less than 10 copies/ 10^6 white blood cells (WBC) (Table 3). Finally, very similar results were obtained using methods 1 and 2.

Table 3
Application 2: amounts of HIV-1 DNA in whole blood

Patient	Copies/ 10^6 WBC ^a		Copies/ 10^6 WBC ^b			
	Conventional	Highly sensitive		Conventional	Highly sensitive	
		Method 1	Method 2		Method 1	Method 2
1	<dl ^c	8	7	<dl	<dl ^d	<dl
2	<dl	262	280	<dl	241	249
3	<dl	54	57	<dl	42	48
4	<dl	9	12	<dl	<dl	<dl
5	<dl	133	139	<dl	93	107

^a DNA extracted from 100 μ l volume of whole blood.

^b DNA extracted from 50 μ l volume of whole blood.

^c Detection limit of conventional real-time PCR was 500 copies/ 10^6 cells.

^d Detection limit of highly sensitive real-time PCR was 5 copies/ 10^6 cells.

3.1.5. Application 3: retrospective analysis of HIV-1 DNA levels during HAART

Fig. 4 shows a retrospective analysis of intracellular HIV-1 DNA levels in patient samples collected during 3 years of HAART. Buffy coat samples that had been frozen for more than 3 years at -80°C were analyzed by highly sensitive real-time PCR using method 1. The VL and amounts of HIV-1 DNA before the start of HAART were 230,000 copies/ml and 1310 copies/ 10^6 WBC, respectively. WBC numbers in buffy coat cells were estimated using the $\beta 2\text{M}$ copy number. Eight months after HAART, the viral load decreased below detection levels (<50 copies/ml), and the levels of HIV-1 DNA decreased to 478 copies/ 10^6 cells. Although the viral load was consistently under the detection level for the next 2 years of HAART, the levels of HIV-1 DNA remained at 450 copies/ 10^6 cells.

4. Discussion

In this study, we developed a new estimation method for quantifying intracellular HIV-1 DNA by introducing a pre-quantitation PCR prior to conventional real-time PCR. We developed two methods to quantify HIV-1 DNA with this technique: method 1 utilizes the rate of $\beta 2\text{M}$ gene amplification during the pre-quantitation PCR; and method 2 utilizes a calibration curve based on the crossing point of real-time PCR and standard HIV-1 plasmid concentrations as described previously (O'Doherty et al., 2002). Using either method, we could reproducibly and accurately measure as few as two copies of HIV-1 DNA/ 10^6 cells of HIV-1 DNA. We applied these methods to three trials: (1) quantitation of HIV-1 DNA levels in purified CD4-positive T lymphocytes from patients responding well to HAART; (2) determination of the necessary amount of whole blood needed to quantify HIV-1 DNA; and (3) longitudinal analysis of HIV-1 DNA levels during HAART using frozen buffy coat samples.

In the first of these applications, we measured the level of HIV-1 DNA in the peripheral CD4-positive T lymphocytes of 30 patients responding well to HAART. In all 30 cases, the cells were positive for HIV-1 DNA. There was a wide distribution of HIV-1 DNA copy number, from 2 to 5960 copies/ 10^6 cells. This distribution was quite similar to previous reports (Andreoni et al., 2000; Chun et al., 1997, 2003; Désiré et al., 2001). In four patients (27, 28, 29, and 30), the level of HIV-1 DNA was quite low. However, based on nested-PCR sequencing and real-time PCR fluorograms, these were clearly not artificially low. In this study, HIV-1 DNA could be quantified in all of the samples using our highly sensitive method, whereas the conventional method could quantify HIV-1 DNA levels in 60% of the samples. Thus, our highly sensitive method using the new estimation technique is an improvement over the conventional method.

In the second application, we determined the amount of whole blood needed to quantify HIV-1 DNA using our highly

sensitive method. We found that 100 μl whole blood is sufficient.

In the third application, we retrospectively examined the change in intracellular HIV-1 DNA levels during 3 years of HAART by using frozen buffy coat samples. The level of HIV-1 DNA before HAART was 1310 copies/ 10^6 WBC. After 8 months of HAART, when the plasma viral load was suppressed below the detection levels (50 copies/ml), the HIV-1 DNA level was 478 copies/ 10^6 WBC. During the next 2 years of HAART, the viral load was maintained under the detection level, and the HIV-1 DNA level remained close to 450 copies/ 10^6 WBC. The decline of HIV-1 DNA in the first phase was 0.45 log. Although this was much less than the decline in plasma viral load, this result was similar to previous reports (Désiré et al., 2001; Ibanez et al., 1999; Izopet et al., 1998; Ngo-Giang-Huong et al., 2001; Perelson et al., 1997; Riva et al., 2001). Finally, we were able to determine the level as copy numbers of HIV-1 DNA/ 10^6 WBC by simultaneously measuring the $\beta 2\text{M}$ copy numbers in the measured samples. Taken together, the new estimation method for highly sensitive quantitation of HIV-1 DNA developed here is useful for estimating the HIV-1 reservoirs in peripheral CD4-positive T lymphocytes of patients responding well to HAART.

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Conventional HPLC Method Used for Simultaneous Determination of the Seven HIV Protease Inhibitors and Nucleoside Reverse Transcription Inhibitor Efavirenz in Human Plasma

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We developed a simple HPLC method for the simultaneous quantitative determination of seven HIV protease inhibitors: amprenavir (APV), atazanavir (ATV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV), and a nucleoside reverse transcription inhibitor, efavirenz (EFV). This method involves a rapid liquid-liquid drug extraction from plasma, the use of an isocratic elution on a reversed-phase C₁₈ column, and an ultraviolet detection at a single wavelength (205 nm). The mobile phase consisted of 39% 50 mM phosphate buffer (pH 5.9), 22% methanol and 39% acetonitrile. Forty-eight samples could be measured in one day since the runtime of one sample is 30 min. The assay has been validated over a concentration range of 0.05 to 12.20 µg/ml for APV, 0.09 to 12.05 µg/ml for ATV, 0.05 to 12.01 µg/ml for IDV, 0.12 to 12.36 µg/ml for LPV, 0.18 to 12.20 µg/ml for NFV, 0.12 to 12.33 µg/ml for RTV, 0.12 to 12.06 µg/ml for SQV, and 0.05 to 12.17 µg/ml for EFV. Calibration curves were linear in the described concentration ranges. The average accuracy ranged from 97.2 to 106.8%. Both the interday and intraday coefficients of variation for all drugs tested were less than 8.5%. This method provides a simple, accurate, and precise method for the therapeutic drug monitoring of the seven protease inhibitors and EFV in clinical routine use.

Key words HPLC; therapeutic drug monitoring; HIV protease inhibitor; human immunodeficiency virus (HIV)-1

The clinical treatment of patients with human immunodeficiency virus (HIV)-1 infection has been advanced by the development of highly active antiretroviral therapy (HAART). HAART reduces plasma HIV-RNA below detectable limits in most cases. However, some patients do not have a sustainable antiviral response, even after experiencing a decrease in plasma HIV-RNA, due to the development of drug resistance and metabolic complications. This undesirable outcome may result from a failure to achieve effective antiretroviral drug plasma concentrations. Therefore, monitoring plasma drug concentrations is essential to ensure optimal drug efficacy, to prevent viral resistance, to manage drug interactions, to avoid adverse effects, and to assess nonadherence.

In recent years several HPLC methods for simultaneous determination of antiretroviral drugs in plasma have been published.¹⁻¹⁵ However, to popularize the simultaneous determination method, a simplified technique is necessary because the reported techniques require a solid-phase extraction,⁴⁻⁸ and/or use of a gradient elution,⁶⁻¹³ and/or an ultraviolet detection at multi wavelengths,^{4,5,7-9,11,13-15} all of which are not routinely available in conventional hospital laboratories. Therefore, we aimed to develop a simple procedure for simultaneous quantitative determination of seven protease inhibitors (PI): amprenavir (APV), atazanavir (ATV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV), and the nucleoside reverse transcription inhibitor, efavirenz (EFV), in human plasma. Our technique involves rapid liquid-liquid drug extraction from plasma, the use of an isocratic elution, as well as an ultraviolet detection at a single wavelength. This assay is based on our previously published HPLC method.¹⁶

MATERIALS AND METHODS

Chemicals and Reagents APV was kindly provided by Glaxosmithkline Research and Development, Ltd. (Park Road, Ware, U.K.), ATV was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (New Brunswick, NJ, U.S.A.), and IDV and EFV were provided by Merck & Co., Inc. (Rahway, NJ, U.S.A.). LPV, RTV and the internal standard (IS), (5S,8S,10S,11S)-9-hydroxy-2-cyclopropyl-5-(1-methylethyl-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatriodecan-13-oic acid, 5-thiazolylmethyl ester, were generously provided by Abbott Laboratories (Abbott Park, IL, U.S.A.). NFV was provided by Agouron Pharmaceuticals (La Jolla, CA, U.S.A.) and SQV was provided by Roche Products (Welwyn Garden City, U.K.). Acetonitrile, methanol, ethyl acetate and *n*-hexane (Kanto Chemical, Tokyo, Japan) were HPLC grade. Sodium carbonate was purchased from Katayama Chemical (Osaka, Japan). Water was deionized and osmosed using a Milli-Q[®] system (Millipore, Bedford, MA, U.S.A.).

Chromatography The HPLC system consisted of a Waters pump (model 515), WISP 712 auto sample processor, and a 2487 dual absorbance detector coupled to the Millennium³² version 3.21 software (Waters, Milford MA, U.S.A.). The analytical column was a Radial-Pak Nova-Pak C₁₈ column (4 µm, 8×100 mm, Waters) protected by Guard-Pak Inserts Nova-Pak C₁₈ precolumn. Absorbance was measured at 205 nm and separations were performed at 30 °C. The mobile phase consisted of 39% 50 mM phosphate buffer (pH 5.9), 22% methanol and 39% acetonitrile. The assay run time was 30 min with a flow rate of 1.8 ml/min. Drugs were quantified by measuring the peak areas under the chromatograms.

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Standard Solutions Stock solutions of all seven protease inhibitors and EFV were prepared by dissolving accurately weighed amounts of each reference compound in water/ethanol (50:50, v/v) to yield concentrations of 106.1 $\mu\text{g/ml}$ for APV, 502.0 $\mu\text{g/ml}$ for ATV, 100.1 $\mu\text{g/ml}$ for IDV, 95.1 $\mu\text{g/ml}$ for LPV, 305.0 $\mu\text{g/ml}$ for NFV, 425.0 $\mu\text{g/ml}$ for RTV, 67.0 $\mu\text{g/ml}$ for SQV, and 93.6 $\mu\text{g/ml}$ for EFV. These stock solutions were stored at -80°C and thawed on the day of analysis. Each stock solution was diluted in drug-free plasma to yield concentrations of 0.05, 0.85, 2.12, 7.43 and 12.20 $\mu\text{g/ml}$ for APV, 0.09, 0.88, 2.20, 6.02 and 12.05 $\mu\text{g/ml}$ for ATV, 0.05, 0.80, 2.00, 6.01 and 12.01 $\mu\text{g/ml}$ for IDV, 0.12, 0.95, 1.90, 6.18 and 12.36 $\mu\text{g/ml}$ for LPV, 0.18, 0.92, 3.05, 6.10 and 12.20 $\mu\text{g/ml}$ for NFV, 0.12, 0.86, 1.71, 6.16 and 12.33 $\mu\text{g/ml}$ for RTV, 0.12, 0.92, 3.05, 6.10 and 12.06 $\mu\text{g/ml}$ for SQV, and 0.05, 0.84, 1.87, 6.08 and 12.17 $\mu\text{g/ml}$ for EFV.

Sample Preparation Two milliliters of ethyl acetate/n-hexane (50:50, v/v) containing the IS (2.02 $\mu\text{g/ml}$) and 1 ml of 0.5 M sodium carbonate were added to a 500 μl plasma sample. The mixture was vortexed and then centrifuged at $3500\times g$ for 5 min. The organic layer was separated and

evaporated dry. The dried material was then dissolved in 100 μl of a mobile phase solution and centrifuged at $13000\times g$ for 5 min. Lastly, 25 μl of the upper solution was injected into the HPLC column. Before taking peripheral blood, written informed consent was obtained from all patients and a healthy volunteer.

Validation Intraday and interday precision values using this method were estimated by assaying control plasma containing five different concentrations of APV, ATV, IDV, LPV, NFV, RTV, SQV and EFV five times on the same day and on three separate days to obtain the coefficient of variation (CV). Accuracy was determined as the percentage of the nominal concentration. Drug recovery from plasma was evaluated by analyzing triplicate samples with or without extraction.

RESULTS

Chromatograms of Plasma Sample Figure 1A shows the chromatogram of a plasma sample containing 2.12 $\mu\text{g/ml}$ of APV, 2.20 $\mu\text{g/ml}$ of ATV, 2.00 $\mu\text{g/ml}$ of IDV, 1.90 $\mu\text{g/ml}$ of LPV, 3.05 $\mu\text{g/ml}$ of NFV, 1.71 $\mu\text{g/ml}$ of RTV, 3.05 $\mu\text{g/ml}$ of

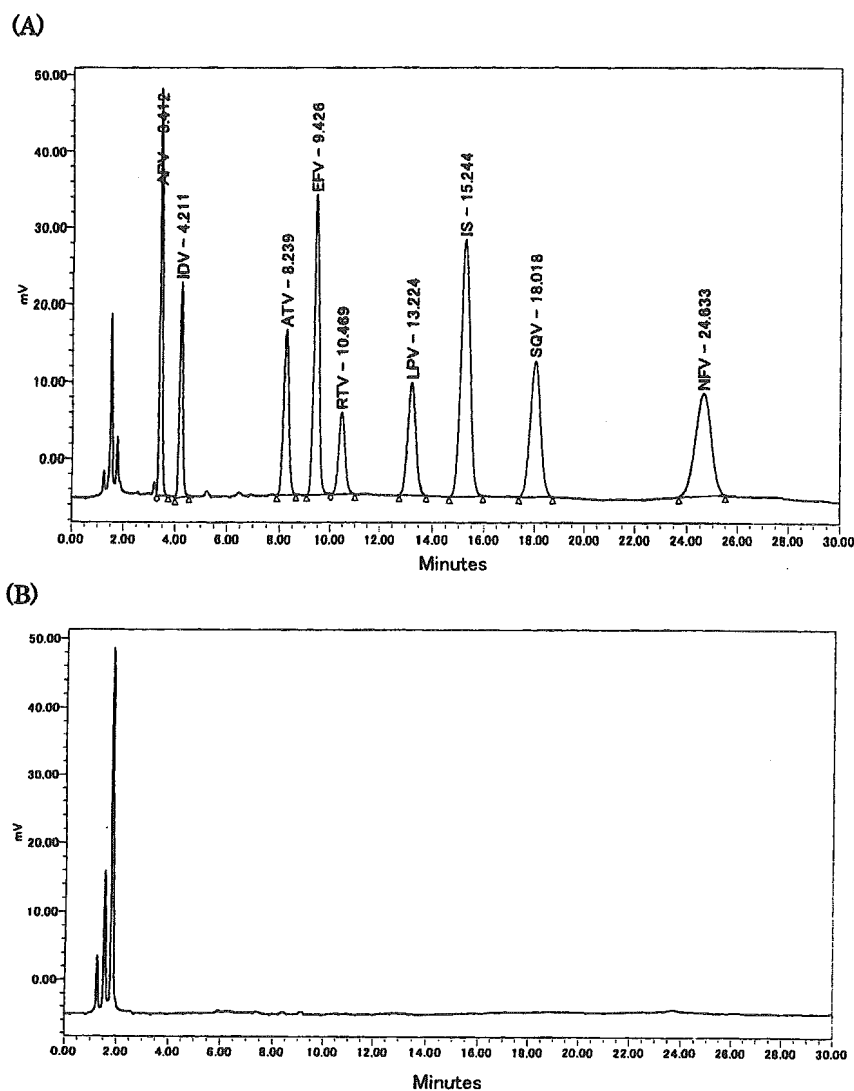


Fig. 1. Chromatograms Obtained after Extraction of (A) Spiked Plasma Sample Containing 2.12 $\mu\text{g/ml}$ of APV, 2.20 $\mu\text{g/ml}$ of ATV, 2.00 $\mu\text{g/ml}$ of IDV, 1.90 $\mu\text{g/ml}$ of LPV, 3.05 $\mu\text{g/ml}$ of NFV, 1.71 $\mu\text{g/ml}$ of RTV, 3.05 $\mu\text{g/ml}$ of SQV and 1.87 $\mu\text{g/ml}$ of EFV and (B) Drug-Free Human Plasma Sample from a Healthy Volunteer.

Table 1. Intraday and Interday Precision and Accuracy for Seven PIs and EFV

	Expected ($\mu\text{g/ml}$)	Intraday ($n=5$)		Interday ($n=15$)		Accuracy (%)	Recovery (%)
		Measured ($\mu\text{g/ml}$)	CV (%)	Measured ($\mu\text{g/ml}$)	CV (%)		
APV	0.05	0.05 \pm 0.01	6.5	0.05 \pm 0.01	5.2	97.8 \pm 5.1	98.4 \pm 3.3
	0.85	0.85 \pm 0.02	2.7	0.85 \pm 0.03	3.0	99.7 \pm 3.0	90.9 \pm 4.4
	2.12	2.14 \pm 0.03	1.4	2.12 \pm 0.04	2.0	100.1 \pm 2.0	91.1 \pm 2.6
	7.43	7.48 \pm 0.12	1.6	7.45 \pm 0.18	2.4	100.3 \pm 2.4	90.1 \pm 1.0
	12.20	12.27 \pm 0.32	2.6	12.25 \pm 0.28	2.3	100.4 \pm 2.3	94.0 \pm 2.6
ATV	0.09	0.09 \pm 0.01	4.8	0.09 \pm 0.01	4.1	98.4 \pm 4.0	92.2 \pm 1.7
	0.88	0.88 \pm 0.05	5.6	0.86 \pm 0.04	5.0	98.1 \pm 4.9	93.4 \pm 3.3
	2.20	2.26 \pm 0.03	1.2	2.22 \pm 0.06	2.6	100.9 \pm 2.6	94.4 \pm 2.5
	6.02	6.04 \pm 0.12	1.9	5.95 \pm 0.15	2.6	98.8 \pm 2.5	91.1 \pm 1.0
	12.05	12.08 \pm 0.26	2.1	12.29 \pm 0.25	2.0	102.0 \pm 2.1	95.0 \pm 2.3
IDV	0.05	0.05 \pm 0.01	5.4	0.05 \pm 0.01	7.5	101.8 \pm 7.7	83.4 \pm 4.4
	0.80	0.79 \pm 0.02	2.8	0.82 \pm 0.05	5.7	102.5 \pm 5.8	96.0 \pm 5.0
	2.00	2.01 \pm 0.04	1.8	2.00 \pm 0.03	1.4	100.1 \pm 1.4	82.6 \pm 2.8
	6.01	5.93 \pm 0.11	1.8	5.93 \pm 0.13	2.2	98.8 \pm 2.2	81.2 \pm 2.3
	12.01	11.96 \pm 0.29	2.5	12.15 \pm 0.26	2.2	101.2 \pm 2.2	85.6 \pm 1.4
LPV	0.12	0.13 \pm 0.01	6.6	0.13 \pm 0.01	7.0	102.7 \pm 7.2	97.5 \pm 8.5
	0.95	0.97 \pm 0.02	2.2	0.95 \pm 0.03	3.4	99.7 \pm 3.4	93.8 \pm 5.7
	1.90	1.98 \pm 0.02	1.2	1.95 \pm 0.04	2.3	102.3 \pm 2.3	93.9 \pm 1.1
	6.18	6.20 \pm 0.09	1.4	6.09 \pm 0.15	2.5	98.5 \pm 2.5	92.0 \pm 1.1
	12.36	12.62 \pm 0.36	2.9	13.14 \pm 0.49	3.8	106.3 \pm 4.0	95.2 \pm 2.4
NFV	0.18	0.18 \pm 0.02	8.5	0.18 \pm 0.01	8.4	97.2 \pm 8.2	96.4 \pm 8.3
	0.92	0.94 \pm 0.03	3.1	0.93 \pm 0.06	6.3	101.2 \pm 6.4	92.7 \pm 5.7
	3.05	3.10 \pm 0.02	0.6	3.09 \pm 0.07	2.2	101.4 \pm 2.3	88.6 \pm 2.1
	6.10	6.12 \pm 0.16	2.7	6.02 \pm 0.22	3.7	98.7 \pm 3.7	89.6 \pm 2.0
	12.20	12.41 \pm 0.46	3.7	12.58 \pm 0.35	2.8	103.1 \pm 2.9	92.8 \pm 2.2
RTV	0.12	0.11 \pm 0.01	7.8	0.12 \pm 0.01	7.5	98.7 \pm 7.4	94.2 \pm 9.9
	0.86	0.85 \pm 0.02	2.5	0.89 \pm 0.04	4.9	103.8 \pm 5.0	90.4 \pm 1.8
	1.71	1.72 \pm 0.06	3.2	1.75 \pm 0.06	3.4	102.2 \pm 3.5	90.4 \pm 3.3
	6.16	6.15 \pm 0.21	3.4	6.12 \pm 0.14	2.3	99.3 \pm 2.3	90.4 \pm 2.1
	12.33	12.27 \pm 0.32	2.6	12.55 \pm 0.31	2.5	101.8 \pm 2.5	94.4 \pm 1.9
SQV	0.12	0.13 \pm 0.01	6.4	0.12 \pm 0.01	6.4	101.4 \pm 6.5	91.0 \pm 5.4
	0.92	0.89 \pm 0.02	1.9	0.92 \pm 0.03	3.6	100.4 \pm 3.6	89.9 \pm 3.4
	3.05	3.11 \pm 0.01	0.4	3.08 \pm 0.08	2.7	101.0 \pm 2.7	91.2 \pm 2.1
	6.10	6.05 \pm 0.16	2.7	5.99 \pm 0.17	2.8	98.3 \pm 2.7	90.2 \pm 1.5
	12.06	12.46 \pm 0.55	4.4	12.88 \pm 0.53	4.1	106.8 \pm 4.4	94.1 \pm 2.3
EFV	0.05	0.05 \pm 0.01	3.8	0.05 \pm 0.01	3.5	101.8 \pm 3.6	93.0 \pm 6.1
	0.84	0.87 \pm 0.02	1.8	0.85 \pm 0.06	6.5	101.5 \pm 6.6	95.2 \pm 3.4
	1.87	1.89 \pm 0.04	2.0	1.90 \pm 0.05	2.6	101.4 \pm 2.6	92.0 \pm 2.4
	6.08	6.24 \pm 0.24	3.8	6.10 \pm 0.20	3.3	100.2 \pm 3.4	91.1 \pm 1.1
	12.17	12.19 \pm 0.27	2.2	12.49 \pm 0.33	2.7	102.6 \pm 2.7	95.2 \pm 2.2

SQV, and 1.87 $\mu\text{g/ml}$ of EFV. Under the described chromatographic conditions, retention times were 3.4, 4.2, 8.2, 10.5, 13.2, 15.2, 18.0, 24.6, and 9.4 min for APV, IDV, ATV, RTV, LPV, IS, SQV, NFV, and EFV, respectively. At a detection wavelength of 205 nm, assays performed on drug-free human plasma failed to show the presence of any interfering peaks during the interested intervals of the retention times (Fig. 1B). These results indicated that the established conditions were valid when measuring these drugs simultaneously.

Validation: Linearity, Precision, Accuracy and Recovery Calibration curves of APV, ATV, IDV, LPV, NFV, RTV, SQV and EFV appeared linear in the concentration range of 0.05 to 12.20 $\mu\text{g/ml}$ for APV, 0.09 to 12.05 $\mu\text{g/ml}$ for ATV, 0.05 to 12.01 $\mu\text{g/ml}$ for IDV, 0.12 to 12.36 $\mu\text{g/ml}$ for LPV, 0.18 to 12.20 $\mu\text{g/ml}$ for NFV, 0.12 to 12.33 $\mu\text{g/ml}$ for RTV, 0.12 to 12.06 $\mu\text{g/ml}$ for SQV and 0.05 to 12.17 $\mu\text{g/ml}$ for EFV, with correlations of 1.000, 0.9999, 0.9999, 0.9993, 0.9997, 0.9999, 0.9991, and 0.9999, respectively.

Precision, accuracy, and extraction recovery of our HPLC method are shown in Table 1. The selected concentration of

each drug covers the expected plasma concentrations found in the patients.

The CVs calculated for APV in the intraday and interday assays ranged from 1.4 to 6.5% and 2.0 to 5.2%, respectively. In ATV CVs ranged from 1.2 to 5.6% and 2.0 to 5.0%, and in IDV, CVs ranged from 1.8 to 5.4% and 1.4 to 7.5%. For LPV, CVs ranged from 1.4 to 6.6% and 2.3 to 7.0%, NFV CVs ranged from 0.6 to 8.5% and 2.2 to 8.4%, RTV CVs ranged from 2.5 to 7.8% and 2.3 to 7.5%, SQV CVs ranged from 0.4 to 6.4% and 2.7 to 6.4%, while for EFV the CVs ranged from 2.0 to 3.8% and 2.6 to 6.5%. Accuracies ranged from 97.8 to 100.4%, 98.1 to 102.0%, 98.8 to 102.5%, 98.5 to 106.3%, 97.2 to 103.1%, 98.7 to 103.8%, 98.3 to 106.8% and 100.2 to 102.6% for APV, ATV, IDV, LPV, NFV, RTV, SQV and EFV, respectively.

Drug recoveries from plasma ranged from 90.1 to 98.4%, 91.1 to 95.0%, 81.2 to 96.0%, 92.0 to 97.5%, 88.6 to 96.4%, 90.4 to 94.4%, 89.9 to 94.1% and 91.1 to 95.2% for APV, ATV, IDV, LPV, NFV, RTV, SQV and EFV, respectively. Extraction recovery of the IS was 100%.

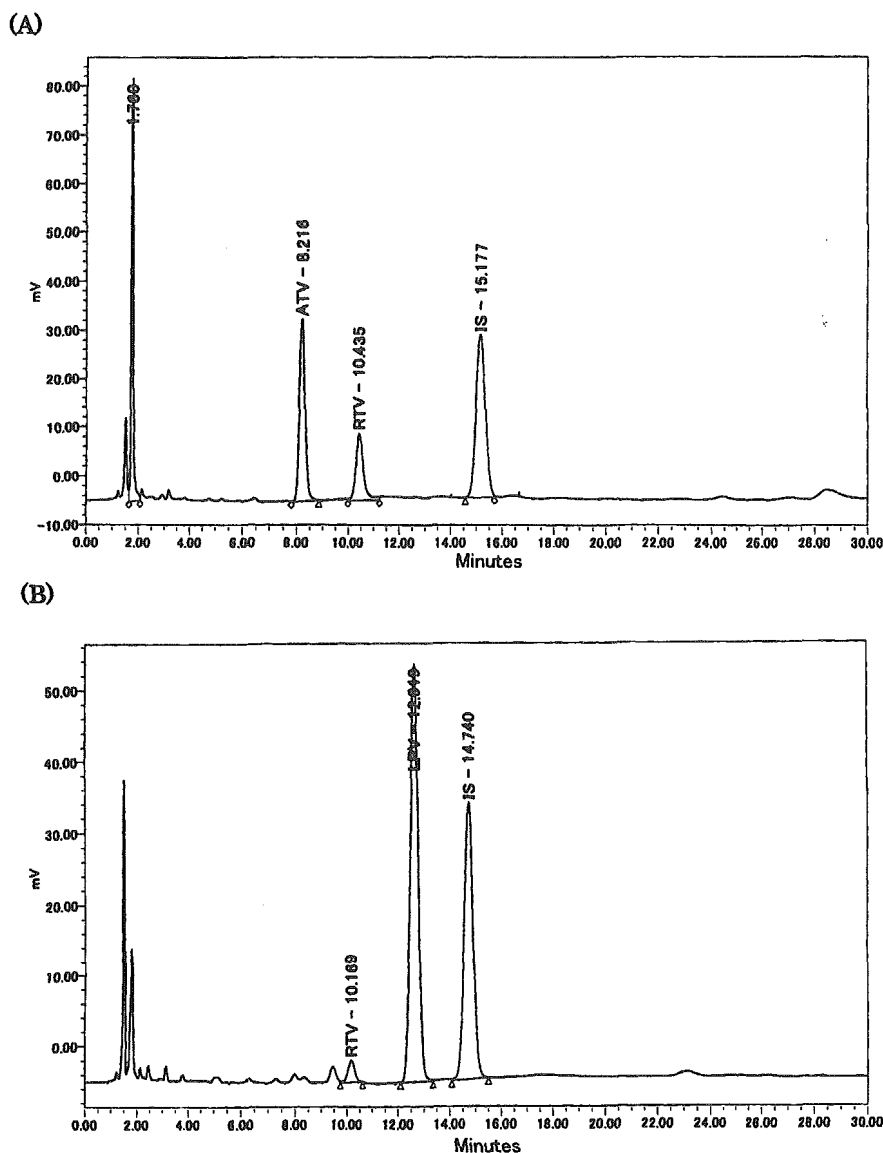


Fig 2. (A) Chromatogram of a Plasma Sample from an HIV-1-Infected Patient Treated with ATV and RTV and (B) Chromatogram of a Plasma Sample from an HIV-1-Infected Patient Treated with LPV and RTV

Chromatograms of Patient Samples Figure 2A shows a chromatogram of a plasma sample from an HIV-1-infected patient treated with ATV and RTV. Fig. 2B shows a chromatogram of a plasma sample from an HIV-1-infected patient treated with LPV and RTV. There is no significant drift from the baseline and no interfering peaks affecting quantification of ATV, LPV and RTV in this chromatogram. These results were validated by peak testing and library matching performed with Millennium³² software.

DISCUSSION

There have been some recent reports of HPLC methods for simultaneous determination of antiretroviral drugs.¹⁻¹⁵⁾ However, these methods have several disadvantages in terms of cost performance, time consumption and necessary equipment; for example, the use of expensive disposable cartridges at the solid-phase drug extraction, gradient elution control by a gradient HPLC pump system, and the ultraviolet detection at multiple wavelengths.

We describe the development, validation, and application of a simple HPLC method for simultaneous quantitative determination of seven PIs currently on the market as well as EFV. The principal advantages of our method are a rapid liquid-liquid drug extraction from plasma, an isocratic elution on a reversed-phase C₁₈ column, and ultraviolet detection at a single wavelength (205 nm).

Generally, the retention time of antiretroviral drugs are dependent on pH of the mobile phase buffer.¹⁴⁾ In fact, some previously reported assays used the mobile phase buffer at a variety of pH values. We sought the optimum pH of the mobile phase buffer by changing pH every 0.5 from pH 2 to pH 11. Finally, we determined pH 5.9 phosphate buffer for the mobile phase to separate each drug. A mobile phase with a pH 9.4 phosphate buffer also enabled excellent drug separation. The overlapping profile of some peaks was not excluded in the pH condition except about 6 and 9.5. However, we recommend pH 5.9 phosphate buffer, because alkaline buffer rapidly degrades the C₁₈ reversed-phase column and causes a baseline drift.

In this study, calibration curves of all the drugs used were linear in the described concentration ranges and the average accuracy ranged from 97.2 to 106.8%. Both interday and intraday CVs for all drugs were less than 8.5%, which is similar to or much lower than previously reported values.^{1–15)} Mean extraction recoveries varied from 81.2% (IDV) to 98.4% (APV). These results indicate that the method developed here achieves a high degree of reproducibility and accuracy.

When antiretroviral drugs are administered at the recommended dose, plasma concentrations are expected in the 0.3 to 8.2 µg/ml range for APV,¹²⁾ the 0.2 to 10 µg/ml range for ATV,¹⁷⁾ the 0.1 to 7.7 µg/ml range for IDV,^{18,19)} the 5.5 to 9.6 µg/ml range for LPV,¹²⁾ the 0.1 to 4.0 µg/ml range for NFV,^{18,20)} the 0.1 to 11.2 µg/ml range for RTV,^{18,20)} the 0 to 0.2 µg/ml range for SQV,¹⁸⁾ and the 1 to 4 µg/ml range for EFV.²¹⁾ Our method successfully covers these regions with good precision and accuracy.

Figure 1 illustrates the chromatogram of a plasma sample containing IS, APV, ATV, IDV, LPV, NFV, RTV, SQV, and EFV. Our isocratic elution yields sharp peaks for all the drugs tested and gives an excellent separation for each. The chromatogram of the blank plasma sample shows that there was no significant drift from the baseline and no interfering peaks affecting the quantification of all the drugs. Furthermore, it turns out that no analytical interference was encountered from endogenous substances or other co-administered drugs at the retention time for each drug, even in the chromatogram of a plasma sample from an HIV-1-infected patient treated with either ATV/RTV or LPV/RTV. Therefore, our method can be made available for optimal follow-up of HIV-infected patients through therapeutic drug monitoring.

In conclusion, this simple HPLC method can be conveniently used as a routine clinical application and enables study of the drug pharmacokinetics in conventional hospital laboratories. This method can also offer continuous measurement of 48 samples in one day.

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PNA-*In Situ* Hybridization Method for Detection of HIV-1 DNA in Virus-Infected Cells and Subsequent Detection of Cellular and Viral Proteins

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Summary

We describe *in situ* hybridization protocols using peptide nucleic acid (PNA) as a probe for detecting HIV-1 DNA in virus-infected cells and the subsequent detection of cellular and/or viral proteins. Because a PNA probe of approx 20 bases was sufficiently long to detect a specific target sequence, a conserved sequence of such a short length was easily identified. Therefore, this probe is valuable even to identify quasi-species of HIV-1. In addition, we adopted a catalyzed signal amplification method to amplify weak viral DNA signals; thus, stringent washing was crucial for eliminating false-positive signals. Our double-staining method using PNA-*in situ* hybridization and subsequent immunostaining enabled the active and inactive proviruses to be distinguished.

Key Words: *In situ* hybridization; peptide nucleic acid; catalyzed signal amplification; HIV-1 provirus; CD4-positive T lymphocytes; p24; HLA-DR.

1. Introduction

In situ hybridization (ISH) is now popularly used in cytogenetic studies to determine the localization of a specific gene on a chromosome and to detect mRNA expression and viral infection within cells using DNA or RNA probes. Nonradioactive ISH methods using fluorescence or visible light to visualize signals generally are used for the detection of target nucleic acid sequences. In general, probes of more than 500 bp in length are required in such studies (1).

Until recently, the detection of HIV-1 has been performed by using autoradiography, using probes labeled with radioisotopes, such as ³⁵S (2-4) and ¹²⁵I (2,5). Although the use of radioactive ISH for the detection of HIV-1 is time consuming and not very convenient, the small copy number of HIV-1 in

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infected cells has hindered the development of a conventional nonradioactive detection system. In addition, designing a suitable proper probe with a length of more than 150 bp is very difficult because of the general lack of long-conserved DNA sequences in viruses, such as HIV-1, that have error-prone reverse transcriptase without any repair activity. To bypass this problem, we developed a peptide nucleic acid (PNA) that mimics the DNA configuration (6–8). PNA, being electrically neutral, can penetrate into cells more easily than DNA and, in addition, PNA can more strongly hybridize with DNA than DNA (9,10). For these reasons, a PNA probe of approx 20 bases in length is long enough to detect a specific target sequence (11).

To overcome the weak viral DNA signal, one of the following methods for signal amplification can be used: one is *in situ* polymerase chain reaction (PCR [12]), and the other is a catalyzed signal amplification (CSA) using biotinyl tyramide (13). *In situ* PCR is quite powerful for amplification of rare target DNA within cells; therefore, *in situ* PCR-driven ISH would be suitable for detecting low copy number DNA sequences. However, this method carries the risk of amplicons synthesized *in situ* diffusing and resulting in false-positives (14). On the other hand, a single copy of the HPV-16 virus was detected successfully using the CSA method (14). Therefore, we adopted the CSA method for detecting HIV-1 DNA (15) and incorporated a crucial stringent washing step to eliminate nonspecific signals that arise from the CSA procedure.

Here, we describe the procedures for detecting HIV-1 DNA in infected CD4-positive T lymphocytes, and the phenotypic determination of HIV-1 DNA-positive cells by a double staining method.

2. Materials

2.1. Cell Lines

1. ACH2: positive control. Human lymphoid cell line latently infected with HIV-1.
2. MOLT4-IIIIB: positive control. Human leukemic cell line persistently infected with HIV-1 strain IIIIB.
3. MOLT4: negative control.

2.2. Sample Preparation

2.2.1. CD4-Positive T-Lymphocytes Smears

1. StemSep column chromatography (Stem Cell Technologies, Inc., Vancouver, BC, Canada; cat. no. STS-14032).
2. Phosphate-buffered saline (PBS) with 3% fetal bovine serum.
3. 4% paraformaldehyde (PFA) containing 0.1 M sodium phosphate buffer, pH 7.4.
4. Ethanol.
5. Silane-coated slides.

6. Wax pen (DakoCytomation A/S, Glostrup, Denmark).
7. Staining racks and containers.
8. Dryer.

2.2.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Tissue blocks of 20% formalin-fixed or 4% PFA-fixed, paraffin-embedded samples.
2. Silane-coated slides.
3. Staining racks and containers.
4. Xylene.
5. Rehydration series: 100, 95, 90, and 70% ethanol.

2.3. Pretreatment

2.3.1. CD4-Positive T Lymphocytes Smears

1. Tris-buffered saline containing Tween-20 (TBST): 50 mM Tris-HCl, 300 mM NaCl, 0.1% Tween-20, pH 7.6.
2. Target retrieval solution (DakoCytomation A/S; cat. no. S1700).
3. Methanol containing 0.3% H₂O₂.
4. Water bath.
5. Staining racks and plastic containers.

2.3.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. The same as **Subheading 2.3.1.**, items 1–5.
2. Proteinase K (DakoCytomation A/S, S3004).

2.4. Preparation of PNA Probe

1. PNA probe: the structure of the probe is as follows: FITC-HN-GCAGCTTCCT-CATTGATGG-CONH₂ (FASMAC Co. Ltd., Kanagawa, Japan; *see Note 1*).
2. DNA ISH solution (DakoCytomation A/S; cat. no. S3305).
3. Cover slips.

2.5. Heat Denaturation

1. Hotplate.

2.6. Hybridization

1. Stringent wash solution (DakoCytomation A/S; cat. no. K5201).
2. TBST.
3. Incubator.
4. Plastic containers.
5. Water bath.
6. Moist chamber.

2.7. PNA Probe Detection by CSA

1. Horseradish peroxidase (HRP)-conjugated anti-FITC antibody (DakoCytomation A/S; cat. no. P5100).
2. Biotinyl tyramide solution (DakoCytomation A/S, GenPoint kit K0620).
3. HRP-conjugated streptavidin (DakoCytomation A/S, GenPoint kit K0620).
4. Alexa Fluor 488-labeled streptavidin (Invitrogen, Carlsbad, CA).
5. 4,6-diamino-2-phenylindole (DAPI) II (Vysis, Inc., Downers Grove, IL).
6. TBST.
7. Staining racks and containers.
8. Cover slips.

2.8. PNA-ISH and Immunohistochemistry (IHC) (Indirect Method)

1. Mouse anti-human CD4 monoclonal antibody (Novocastra Laboratories, Ltd., Newcastle, UK; cat. no. NCL-CD4-1F6) or mouse anti-human HLA-DR monoclonal antibody (DakoCytomation A/S; cat. no. M0746).
2. Alexa Fluor 594-labeled goat anti-mouse IgG antibody (Invitrogen; cat. no. A-11005).
3. DAPI II (Vysis, Inc.).
4. Cover slips.

2.9. PNA-ISH and IHC (Labeled Streptavidin-Biotin Method)

1. Avidin solution (DakoCytomation A/S; cat. no. X0590).
2. Biotin solution (DakoCytomation A/S; cat. no. X0590).
3. Mouse monoclonal anti-HIV-1 p24 antibody (DakoCytomation A/S; cat. no. M0857).
4. Biotinylated goat anti-mouse Ig antibody (DakoCytomation A/S; cat. no. E0433).
5. Alexa Fluor 594-labeled streptavidin (Molecular Probes, Inc.; cat. no. S-11227).
6. DAPI II (Vysis, Inc.).
7. Cover slips.

3. Methods

3.1. Sample Preparation (see Note 2)

3.1.1. CD4-Positive T Lymphocytes Smears

1. Negatively select and purify CD4-positive T lymphocytes by StemSep column chromatography according to the manufacturer's instructions.
2. Spin down the collected cells at 250g for 5 min.
3. Discard supernatant and resuspend with PBS.
4. Mark the area for sample-mount on a silane-coated slide with a wax pen. Drop 5 μ L of the cell suspension onto the slide and spread it out gently using the pipet tip.
The area of the sample-mount (15 \times 15 mm).
5. Dry the slides using a dryer at a cool setting.
6. Fix the slides with 4% PFA containing 0.1 mol/L sodium phosphate buffer, pH 7.4, at room temperature for 60 min or at 4°C overnight.

7. Rinse the slides in PBS (3 min, three times).
8. Dehydrate the slides in absolute ethanol and then store at -20°C until use.

3.1.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Place a 4- to 5- μm section onto a slide. Heat the slide to melt the paraffin in a 60°C oven for 15 min and dry at 37°C overnight.
2. Deparaffinize sections in fresh xylene (3 min, three times) and rehydrate in graded (100, 100, 95, 90, and 70%) ethanols and autoclaved water.

3.2. Pretreatment

3.2.1. CD4-Positive T-Lymphocyte Smears

1. Immerse the slides in autoclaved water for 5 min.
2. Immerse the slides in preheated target retrieval solution for 40 min at 95°C , and allow to cool for 20 min.
3. Wash the slides in autoclaved water (1 min, three times).
4. Immerse the slides in methanol containing 0.3% H_2O_2 for 20 min.
5. Wash the slides in autoclaved water for 1 min.
6. Briefly immerse slides in 95% ethanol and allow to air dry.

3.2.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Immerse the slides in autoclaved water for 5 min.
2. Immerse the slides in preheated target retrieval solution for 40 min at 95°C and allow to cool for 20 min.
3. Wash the slides in autoclaved water (1 min, three times).
4. Digest sections with proteinase K for 10 min at room temperature (*see Note 3*).
5. Wash the slides in autoclaved water (1 min, three times).
6. Treat the slides with methanol containing 0.3% H_2O_2 for 20 min.
7. Wash the slides in autoclaved water for 1 min.
8. Briefly immerse the slides in 95% ethanol and allow to air-dry.
9. Mark the area of the section with a wax pen.

3.3. Preparation of PNA Probe

1. Dilute FITC-conjugated PNA probe in hybridization solution to a final concentration of between 0.2 and 0.5 $\mu\text{g}/\text{mL}$.
2. Apply 25 μL of hybridization solution containing PNA probe to the marked area of the slide.
3. Carefully apply the cover slip, avoiding the introduction of air bubbles.

3.4. Heat Denaturation

1. Heat the slides at 93°C for 5 min on a hotplate to denature the double-stranded DNA.

3.5. Hybridization

1. Incubate the slides with the PNA probe at 45°C for 60–90 min in a moist chamber.
2. After hybridization, immerse the slides in TBST and gently remove the cover slips.

3. Wash the slides in prewarmed stringent wash solution at 57°C (20 min, twice).
4. Immerse the slides in TBST at room temperature for 5 min.

3.6. PNA Probe Detection by CSA (see Notes 4 and 5; Fig. 1)

1. Incubate HRP-conjugated rabbit anti-FITC antibody (1:500 dilution) for 60 min (see Note 6).
2. Wash the slides in TBST (3 min, three times).
3. Incubate biotinyl-tyramide for 15 min.
4. Wash the slides in TBST (3 min, three times).
5. Incubate HRP-conjugated streptavidin (1:600–800 dilution) for 15 min.
6. Wash the slides in TBST (3 min, three times).
7. Incubate biotinyl-tyramide for 15 min.
8. Wash the slides in TBST (3 min, three times).
9. Incubate 0.5 µg/mL Alexa Fluor 488-labeled streptavidin for 15 min in the absence of light.
10. Wash the slides in TBST in the absence of light (3 min, three times).
11. Immerse the slides in distilled water.
12. Apply DAPI II and mount cover slip.

3.7. Fluorescence Microscopy

The slides were examined under a fluorescence microscope (BX50 and BX-FLA, Olympus Corp., Tokyo, Japan) equipped with appropriate filter sets (61002, Chroma Technology Corp., Rockingham, VT). Photographic images of the fluorescent signals were taken with a CCD camera (SenSys 0400, Photometrics Ltd., Tucson, AZ) and were uploaded to a microcomputer using IPLab software (Scanalytics, Inc., Fairfax, VA). The stored images were merged to reveal various aspects (see Note 7; Fig. 2).

3.8. Subsequent Determination of Phenotypes After PNA-ISH Treatment

We describe here a double staining method by PNA-ISH and IHC methods for the detection of both HIV-1 DNA and a protein in the same cell, respectively. Proteins can be detected with one of two methods with IHC: an indirect method and a labeled streptavidin-biotin (LSAB) method. For the detection of CD4 or HLA-DR molecules, the conventional indirect method was used. On the other hand, the LSAB method that has higher sensitivity than the indirect method was performed to detect the p24 HIV-1 capsid protein.

Fig. 1. (*opposite page*) Schematic representation of the procedures for using the PNA-probe in the ISH method. The hybridized probe was detected by sequential reactions of the following antibodies and reagents: HRP-conjugated anti-FITC antibody, biotinylated tyramide (first amplification), HRP-labeled streptavidin, biotinylated tyramide (second amplification), and streptavidin-conjugated Alexa 488.