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Quantitation of Human Immunodeficiency Virus Type 1 DNA and RNA by a Novel Internally Controlled PCR Assay

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A novel internally controlled PCR (ICPCR) assay was developed to accurately quantitate human immunodeficiency virus type 1 (HIV-1) DNA and RNA in peripheral blood mononuclear cells and plasma. The ICPCR assay was sensitive and reproducible within a linear range of amplification of 10^0 to 10^3 copies for HIV-1 DNA and 10^1 to 10^4 copies for HIV-1 RNA. The assay detected HIV-1 RNA in plasma and peripheral blood mononuclear cells from all HIV-1 subjects regardless of disease stage. ICPCR was compared with a branched-DNA signal amplification assay for subjects beginning antiretroviral therapy. The reductions in plasma HIV-1 RNA in response to therapy were comparable with the two assays. The ICPCR assay should be useful in monitoring HIV-1 RNA levels both in natural history studies and in clinical trials of antiretroviral agents.

Accurate quantitation of human immunodeficiency virus type 1 (HIV-1) DNA and RNA by PCR in cells and body fluids is now recognized to be an essential tool in studies of HIV-1 pathogenesis (2–4, 6, 8, 13) and antiretroviral drug efficacy (5, 9). Initially, a number of investigators used a semiquantitative PCR assay to measure the levels of viral DNA and RNA in peripheral blood mononuclear cells (PBMC) and plasma (3, 4, 6, 8, 13). In this procedure, serial dilutions of sample DNA or RNA were assayed by PCR for the presence of viral DNA or RNA. The amount of HIV-1 DNA or RNA was calculated by comparing the signal intensity of the amplimer with a standard curve constructed by amplification of known concentrations of HIV-1 DNA or RNA.

Although these semiquantitative PCR assays can provide important information on the relative copy number of HIV-1 DNA or RNA, they are unable to control for the efficiency of reverse transcription and DNA amplification in each sample. Recently, a quantitative competitive RNA PCR (QC PCR) procedure has been reported in which RNA from PBMC or plasma is subjected to co-reverse transcription and coamplification with serially diluted known copies of plasmid-derived HIV-1 RNA carrying a deletion in the target RNA (1, 12). The number of HIV-1 RNA copies in the sample is determined from the point of equivalence of signal intensity between the sample RNA and the dilution series of deletion plasmid RNA. One limitation of this QC PCR procedure is that each sample generates 10 to 12 amplification assay tubes (5 or 6 dilutions, each in duplicate), making the technique cumbersome for large numbers of samples.

In this report, we describe a novel, internally controlled PCR (ICPCR) assay to quantitate HIV-1 Gag DNA and RNA in PBMC or plasma that requires only two reaction tubes per sample. The assay is sensitive and reproducible within a linear range of amplification that is between 10^0 and 10^3 copies for HIV-1 DNA and between 10^1 and 10^4 for HIV-1 RNA.

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For quantitation of HIV-1 DNA by ICPCR, duplicate cell lysates from 1.25×10^5 PBMC (equivalent to 1 μg of DNA measured by cell count) were mixed with 500 copies of a plasmid HIV-1 Gag DNA carrying a 45-bp deletion in the target sequence and then coamplified with Gag primers 101/145 under conditions described previously (4). The deletion in the plasmid HIV-1 Gag sequence does not affect binding of Gag primer pairs 101/145 or probe 102 (7).

For quantitation of HIV-1 RNA by ICPCR, RNA was extracted from PBMC or from HIV particles pelleted from plasma (13,000 × *g* for 2 h at 4°C) with RNA-zol and then treated with DNase as described previously (4). RNA from 5×10^4 PBMC (50 to 100 ng measured spectrophotometrically) or from the equivalent of 25 μl of plasma was mixed in duplicate with 1,000 copies of a plasmid-derived Gag RNA carrying the same 45-bp deletion described above. The RNA samples were co-reverse transcribed with Moloney murine leukemia virus reverse transcriptase using downstream primer 101 and then coamplified with primers 101/145 as described previously (7). Amplification was performed with 28 repeated cycles consisting of denaturation at 94° for 30 s, primer annealing at 55°C for 30 s, and polymerization at 55°C for 30 s. A Gag RNA standard was prepared by in vitro transcription of the HIV-1 Gag deletion plasmid described above. This in vitro-transcribed RNA was purified by polyacrylamide gel electrophoresis. The copy number of the purified Gag RNA was determined by comparing the intensity of its reverse transcription PCR signal with that derived from a known number of purified HIV particles counted by electron microscopy (two RNA copies per particle). The copy number determined by this procedure is within twofold of that determined spectrophotometrically.

A one-third volume of the 100-μl amplification reaction product was hybridized in solution to the ^{32}P-labeled oligonucleotide probe 102 (250,000 to 300,000 cpm per sample) as described previously (14). The oligonucleotide probe-target DNA heteroduplex was resolved by electrophoresis in a 10% polyacrylamide gel. The gel was dried, and the signal intensity of the amplimers derived from the samples and from the exogenously added deletion plasmid DNA or RNA standards was measured with a Phosphorimager. The amount of HIV-1

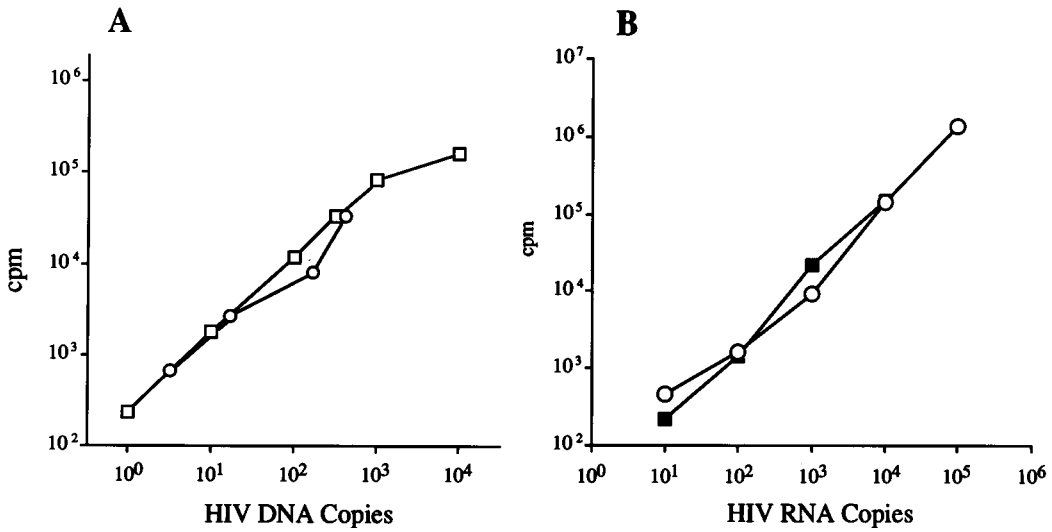

FIG. 1. Input-response amplification profiles for HIV-1 DNA and RNA by ICPCR. (A) HIV-1 DNAs from the deletion plasmid (□) and 8E5 cells (○); (B) HIV-1 RNAs from the deletion plasmid (○) and from purified virus particles (■).

DNA or RNA in the sample was quantitated by interpolation from a standard curve generated for each experiment with known copies of deletion plasmid DNA or RNA by serial 10-fold dilutions of stock plasmid DNA and RNA of known concentrations. This unadjusted copy number was adjusted further as: adjusted copy number = unadjusted copy number × [signal (counts per minute) from the deletion plasmid in the standard curve ÷ signal from the deletion plasmid in the sample]. This adjustment corrected for any depression of the PCR signal that occurred in the co-reverse transcription or coamplification reactions. The extent of signal attenuation of the DNA or RNA standard in coamplifications and co-reverse transcription was always less than twofold. For each sample, a control cDNA synthesis without the reverse transcriptase was performed to ensure that the signal was from HIV-1 RNA and not from any contaminating HIV-1 DNA in the sample. The no-reverse transcriptase control was considered to be negative if the signal was less than the average signal plus 2 standard deviations from two no-RNA controls.

The quantitative branched-DNA (bDNA) signal amplification assay of HIV-1 RNA in plasma (Quantiplex HIV RNA assay; Chiron Corp., Emeryville, Calif.) was performed according to the manufacturer's instructions. The bDNA assay has a quantitation limit of 1×10^4 HIV-1 eq/ml and is linear up to 1.6×10^6 eq/ml (11).

In initial experiments, we defined the linear range of PCR amplification of known copies of wild-type (full-length) and deletion plasmid HIV-1 Gag DNA and RNA. Figure 1A shows the input-response profiles (slopes and sensitivities) for PCR amplification of deletion plasmid HIV-1 Gag DNA and proviral HIV-1 DNA (from 8E5 cells) run in separate reaction tubes. The profiles are highly comparable and linear over a range of 10^0 to 10^3 copies of HIV-1 DNA. Similarly, Fig. 1B shows comparable linear input-response profiles for reverse transcription PCR with 10^1 to 10^4 copies of the HIV-1 deletion plasmid or wild-type RNA (purified from 8E5 cells). These results indicate that the amplification efficiency of the deletion plasmid DNA or RNA is comparable to that of wild-type HIV-1 DNA or RNA.

Next, we examined whether there was competition between plasmid DNA or RNA and sample target DNA or RNA when they are co-reverse transcribed and/or coamplified in the same reaction tube over the linear range of amplification. These experiments demonstrated that the amplification signal from 5 to 625 copies of 8E5 HIV-1 DNA was not reduced by 500 copies of deletion plasmid HIV-1 DNA (data not shown). Similarly, 1,000 copies of the deletion plasmid HIV-1 RNA did not reduce the signal from 10^1 to 10^4 molecules of 8E5 target HIV-1 RNA (data not shown). From these results, we concluded that the number of copies of HIV-1 DNA and RNA could be measured accurately within the linear range of amplification without significant competition between target DNA or RNA and 500 copies of deletion plasmid DNA or 1,000 copies of deletion plasmid RNA, respectively.

The reproducibility of the ICPCR assay was assessed by quantitating in three separate experiments the amounts of HIV-1 DNA in five PBMC samples and HIV-1 RNA in five plasma samples. Figure 2 shows representative ICPCR results for HIV-1 DNA and RNA. The target HIV-1 DNA or RNA signal was easily distinguishable from the deletion plasmid DNA or RNA standard. The results shown in Tables 1 and 2

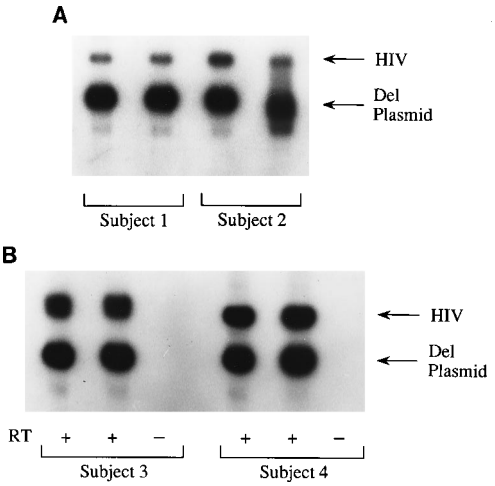

FIG. 2. Autoradiograms of HIV-1 DNA (A) and RNA (B) quantitation by ICPCR. Del, deletion; RT, reverse transcriptase.

TABLE 1. Reproducibility of quantitative HIV-1 DNA PCR

Sample	No. of copies/10 $CD4^+$ cells in expt:		
	I	II	III
1	2,566	2,719	2,780
2	1,148	1,337	1,501
3	19,587	20,374	29,000
4	3,143	3,653	3,688
5	220	363	340

indicate that the reproducibility of the assay is within twofold. The mean coefficients of variation were 31% for HIV-1 RNA and 10% for HIV-1 DNA ($n = 15$).

The ICPCR assay was used to quantitate the HIV-1 DNA and RNA in PBMC from 25 HIV-1-infected subjects enrolled in the Multicenter AIDS Cohort Study. Patients varied in disease stage ranging from A_1 (asymptomatic) to C3 (AIDS) (Centers for Disease Control and Prevention 1993 classification). As shown in Table 3, the number of HIV-1 DNA copies ranged from 6 to 15,561 per million $CD4^+$ cells. HIV-1 RNA copies ranged from 256 to 111,598 copies per million $CD4^+$ T cells. As reported by other investigators using different PCR assays (1, 8, 12), ICPCR could detect HIV-1 RNA in PBMC from subjects at all stages of the disease, including those with high $CD4^+$ cell counts ($>700/mm^3$). As we and others (1, 5, 8, 13) have previously reported, the levels of HIV-1 RNA and DNA in PBMC were inversely correlated with the $CD4^+$ T-cell counts ($P < 0.01$; Spearman rank coefficients of correlation, -0.77 for HIV-1 RNA and -0.65 for HIV-1 DNA).

ICPCR was also applied to measure HIV-1 RNA in plasma from the same 25 subjects. As shown in Table 2, ICPCR detected HIV-1 RNA in plasma from all subjects, and the plasma RNA levels correlated well with the level of cellular HIV-1 RNA. As for cellular HIV-1 RNA, plasma HIV-1 RNA levels were significantly higher in subjects with lower $CD4^+$ cell counts ($P < 0.05$; Spearman rank coefficient of correlation, -0.45).

The ICPCR assay was compared with the bDNA signal amplification assay for the quantitation of HIV-1 RNA in 20 plasma samples. As shown in Table 3, the results of the two assays correlated (Spearman rank coefficient of correlation, 0.91), but ICPCR was more sensitive than the bDNA assay, especially at an RNA concentration of less than 5,000 copies per ml of plasma. However, it is interesting that the number of plasma RNA copies per milliliter detected by the ICPCR was consistently lower than those obtained by the bDNA assay. This could be due to differences in the methods used to quantitate the RNA standards used in these two assays.

Finally, ICPCR was applied to quantitate plasma HIV-1 RNA in subjects beginning experimental antiretroviral therapy as part of a randomized, blinded clinical trial. Plasma samples from four subjects who showed reductions in plasma HIV-1 RNA within 10 weeks of starting drug therapy as measured by the bDNA assay were tested by the ICPCR assay. Figure 3 shows that ICPCR detected changes in the plasma HIV-1 RNA level in response to therapy in all four patients. The

TABLE 2. Reproducibility of quantitative HIV-1 RNA PCR

Sample	No. of copies/ml of plasma in expt:		
	I	II	III
6	1,674	1,220	733
7	15,874	14,111	11,150
8	43,587	26,447	34,641
9	19,278	12,042	8,046
10	5,004	5,984	2,892

TABLE 3. Quantitation of HIV-1 DNA and RNA in PBMC and plasma by ICPCR

Patient (no. of $CD4^+$ cells)	ICPCR			No. of RNA eq/ml of plasma by bDNA assay[a]
	No. of copies/10^6 $CD4^+$ cells		No. of RNA copies/ml of plasma	
	DNA	RNA		
101 (948)	6	362	17	<10,000
102 (887)	580	1,235	1,805	ND
103 (862)	283	341	1,566	<10,000
104 (827)	337	592	2,832	<10,000
105 (769)	966	1,240	9,975	ND
106 (776)	331	385	19,278	26,000
107 (756)	884	1,870	2,261	<10,000
108 (719)	41	1,361	428	<10,000
109 (689)	1,571	1,549	4,928	17,000
110 (633)	20	350	1,674	<10,000
111 (510)	550	1,583	2,250	<10,000
112 (491)	2,496	3,180	9,546	29,000
113 (386)	385	256	922	<10,000
114 (382)	68	2,791	1,927	<10,000
115 (300)	182	820	5,004	<10,000
116 (286)	3,535	4,430	15,873	28,000
117 (286)	8,863	20,207	69,644	87,000
118 (208)	4,400	11,846	8,558	42,000
119 (193)	3,504	17,546	205,231	279,000
120 (134)	12,739	26,075	23,811	43,000
121 (72)	4,819	24,102	57,998	217,000
122 (33)	15,561	111,598	43,586	ND
123 (27)	4,506	38,507	26,484	37,000
124 (22)	11,595	54,194	141,671	ND
125 (17)	4,593	130,142	44,741	ND

[a] Cutoff, 10,000 HIV-1 RNA eq/ml. ND, not done.

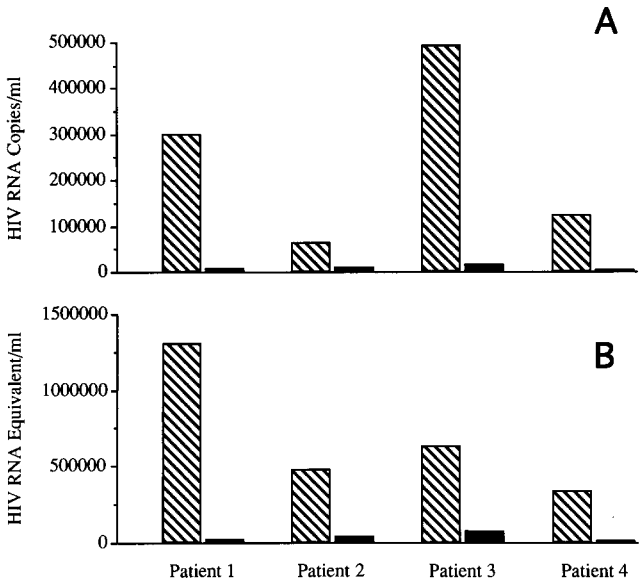

FIG. 3. Comparison of ICPCR (A) and the bDNA assay (B) for quantitating plasma HIV-1 RNA in patients beginning antiretroviral therapy. Hatched and solid bars, levels before and after therapy, respectively.

magnitudes of the observed reduction in HIV-1 RNA measured by the two assays were similar.

This report describes a quantitative ICPCR assay to measure HIV DNA in PBMC and HIV-1 RNA in PBMC and plasma. The assay is reproducible (coefficients of variation, 10 and 31% for HIV-1 DNA and RNA, respectively) and requires only two reaction tubes per sample. The latter advantage makes the assay suitable for large numbers of samples. Furthermore, the assay is sensitive, detecting 10^0 to 10^3 copies of HIV-1 DNA and 10^1 to 10^4 copies of RNA in PBMC or plasma. Samples containing more than 10^3 copies of HIV-1 DNA per 1×10^5 PBMC or 10^4 copies of RNA per 5×10^4 PBMC or 25 μl of plasma can be diluted to fall within the linear range of amplification.

The range of HIV-1 DNA copies (6 to 15,561 per million $CD4^+$ cells) in PBMC measured by ICPCR is close to that (130 to 56,000 per million $CD4^+$ cells) reported by Bagnarelli et al. for QCPCR (1). The ranges of HIV-1 RNA copies in PBMC (256 to 111,598 per million $CD4^+$ cells) and in plasma (17 to 205,231/ml) measured by ICPCR are also similar to those (150 to 230,235 copies per million $CD4^+$ cells and 230 to 762,880 copies per ml of plasma) reported by Bagnarelli et al. for QCPCR (1). Recently, a PCR assay similar to ICPCR, based on colorimetric detection of HIV-1 RNA, has been described by Mulder et al. (10). However, this assay is restricted to the quantitation of HIV-1 RNA in plasma samples and requires special reagents that are not yet commercially available.

In summary, the ICPCR assay can quantitate differences in HIV-1 DNA and RNA levels in plasma and PBMC at different disease stages and after initiation of antiretroviral therapy. ICPCR has the advantage over QCPCR of requiring only two PCRs per sample and, thus, should prove to be useful in monitoring HIV-1 RNA levels in large numbers of samples in both natural history studies and clinical trials of new therapeutic agents.

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