

Rapid and Simple PCR Assay for Quantitation of Human Immunodeficiency Virus Type 1 RNA in Plasma: Application to Acute Retroviral Infection

J. MULDER, N. MCKINNEY, C. CHRISTOPHERSON, J. SNINSKY, L. GREENFIELD, AND S. KWOK*

Department of Infectious Diseases, Roche Molecular Systems, Inc., Alameda, California 94501

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A method for quantitating human immunodeficiency virus type 1 plasma viremia may be useful in monitoring disease progression and the responsiveness of patients to a therapeutic regimen or vaccine. A quantitative assay for viral RNA in plasma or sera that differs in several aspects from those reported previously was developed. First, whereas conventional reverse transcriptase-PCR assays involve a two-step process and use two enzymes, the method described uses a single enzyme, *rTth* DNA polymerase, for both reverse transcription and PCR. The reactions are carried out in a single tube and with a single buffer solution with uninterrupted thermal cycling. Second, uracil-*N*-glycosylase and dUTP are incorporated into the reaction mixtures to ensure that any carryover of DNA from previous amplifications will not compromise quantitation. Third, a quantitation standard is incorporated into each reaction mixture so that differences in amplification efficiency caused by sample interferents, variability in reaction conditions, or thermal cycling can be normalized. To ensure comparable amplification efficiency, the quantitation standard has the same primer-binding regions as the human immunodeficiency virus type 1 target and generates an amplified product of the same size and base composition. The probe-binding region was replaced with a sequence that can be detected separately. Fourth, a colorimetric detection format was modified to provide at least a four-log-unit dynamic range. The quantitative assay requires only a single amplification of the sample and can be completed in less than 8 h. The procedure was used on archival samples to demonstrate the viremic spike in acute infection and the suppressed levels of circulating virus following seroconversion.

The ability to accurately determine viral and infected cell burden is essential in understanding the natural history of human immunodeficiency virus type 1 (HIV-1) infection, predicting disease progression, and assessing the efficacy of various therapeutic drug regimens and vaccines. Although CD4 cell counts are the best-known and most broadly used surrogate marker for AIDS, the level of CD4 cell counts does not always correlate with the disease state. Some HIV-1-infected individuals with very low CD4 cell counts remain healthy, while others with comparatively high levels of CD4 cells experience fulminant disease. Another complicating factor is the variability in determining CD4 cell numbers within and among laboratories. Elevated levels of β_2 -microglobulin, neopterin, and interferon; delayed-type hypersensitivity; and early clinical symptoms have also served as surrogate markers of AIDS (1, 10). It is becoming increasingly clear that the CD4 cell count alone is not sufficient, and investigation of alternative markers is required. In particular, viral load is anticipated to provide insight into the dynamics of the HIV-1 infection and is expected to complement the information provided by CD4 cell counts.

Efforts to quantitate the viral load in infected individuals have been complicated by the paucity of infected cells, viral particles, and expressed viral components. Endpoint dilution cultures have been used to quantify HIV-1 viral particles in plasma and infected peripheral blood mononuclear cells (7, 15). The procedure is laborious, time-consuming, and expensive and requires handling of large quantities of

the infectious agent. Furthermore, cultivation systems inherently select for isolates that are capable of in vitro propagation; noncytopathic or slowly replicating isolates and variants with tropism for other cell types are not detected. Direct detection of the HIV-1 core antigen, p24, in patient sera has been used, but the assay lacks sensitivity, presumably because of both the complexing of the antigen by antibodies and the scarcity of protein in the peripheral blood. Acid dissociation has been used to improve the detection of p24 (19), but this assay still lacks sufficient sensitivity.

The use of PCR (28, 34) to quantitate RNA has been extensively reported (2, 3, 12, 16, 33, 35, 36, 41) and reviewed by Ferre (11) and Clementi et al. (6). The quantitative assay described here offers numerous advantages over conventional reverse transcription (RT) coupled to PCR (RT-PCR) assays. First, whereas some investigators have used labor-intensive methods for virus isolation such as polyethylene glycol precipitation and ultracentrifugation coupled with phenol-chloroform extraction, the sample preparation procedure accompanying this protocol requires only a single guanidinium isothiocyanate (GuSCN) treatment of the plasma and then an isopropanol precipitation step. Second, whereas conventional RT-PCR uses reverse transcriptase from murine leukemia virus or avian myeloblastosis virus with *Taq* DNA polymerase, the procedure described here uses a recombinant DNA polymerase, which was originally isolated from *Thermus thermophilus*, that possesses efficient RT and DNA polymerase activities (29). The use of *rTth* DNA polymerase for RT-PCR eliminates the additional manipulations generally required of two-enzyme systems and has been applied to the detection of hepatitis C virus (HCV) (42). The use of *rTth* DNA polymerase also allows for the incorporation of uracil-*N*-glycosylase (UNG)

* Corresponding author. Mailing address: Department of Infectious Diseases, Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, CA 94501. Phone: 510/814-2820. Fax: 510/814-2997.

for prevention of DNA carryover (22, 26). Third, the sensitivity of the amplification system allows fewer cycles to be used; some procedures require not only higher cycle numbers but also nested amplification. Fourth, the incorporation of an RNA quantitation standard that amplifies equivalently to HIV-1 RNA without compromising amplification of the target is essential in monitoring reaction variability. While several investigators have included quantitation standards, equivalency in the performance of the standard relative to that of the target has not been demonstrated. External standards are most often used; the importance of an internal quantitation standard has not been universally recognized. Fifth, the microwell plate detection assay is easy to use, can be performed rapidly, and provides a quantitative, colorimetric readout (18). Finally, the sensitivity of the amplification and detection system, together with the design of the quantitation standard, allows quantification from a single amplification of the sample being tested. In contrast, the competitive PCR amplification systems require multiple amplifications (3, 12, 33). Furthermore, whereas quantitative strategies that do not use target amplification frequently require 1 ml or more of plasma (40), the equivalent of 50 μ l plasma is used in the assay described here.

We describe here a procedure for quantification of HIV-1 RNA using a single enzyme, *rTth* DNA polymerase, for both RT and PCR. Through optimization, incorporation of a quantitation standard, and validation of the procedure, we demonstrate that the assay is sensitive, reproducible, and quantitative. Furthermore, the ability to incorporate UNG and dUTP into the single-tube amplification eliminates problems associated with PCR product carryover that can compromise quantitation.

MATERIALS AND METHODS

HIV-1 transcript. An approximately 300-base HIV-1 RNA transcript encompassing the SK462-SK431 (18) *gag* primer-binding regions (see below) has been described previously (16). The transcript generated from plasmid pCC2 harbors the HIV-1 SK102 probe-binding region (18) and was used in model studies. Amplification of pCC2 transcripts with SK462-SK431 results in a 142-bp product.

Construction of the quantitation standard. The quantitation standard was a 219-base RNA transcript of a plasmid designated pNAS2. The transcript contains the HIV-1 SK462-SK431 primer-binding sites and generates an amplicon of the same length (142 bp) and base composition as the HIV-1 target. The probe-binding sequence has been rearranged to allow separate microwell detection (25). The quantitation standard was constructed by annealing two synthetic oligonucleotides, one coding for the 5' portion of the positive strand and one coding for the 5' portion of the negative strand. The two oligonucleotides contained eight complementary bases at the 3' termini. The oligomers were annealed on ice for 30 min and were then extended with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of deoxynucleoside triphosphates (dA, dG, dC, dT) at 37°C for 30 min. To facilitate cloning, the upstream primer was designed to generate a *SalI* site in the fully extended product. Following endonuclease cleavage with this enzyme, the fragment was cloned into the *SalI-SmaI* site of plasmid pSP64 [poly(A)]. DNA from the resulting plasmid, pNAS2, was purified, linearized, and transcribed in vitro with SP6 RNA polymerase. The RNA transcripts were treated with RNase-free DNase (Promega, Madison, Wis.), extracted with phenol-chloroform, and passed twice over an

oligo(dT) cellulose column (Stratagene, La Jolla, Calif.). Following ethanol precipitation, the RNA was resuspended in diethylpyrocarbonate (DEPC)-treated water and the concentration was determined by spectrophotometric reading. Transcripts were diluted in DEPC-treated water containing 1 μ g of poly(rA) (Pharmacia, Piscataway, N.J.) per ml.

Sample preparation. Blood was collected into acid citrate glucose tubes, and the plasma was separated by centrifugation at 1,000 \times g for 10 min within 3 h of collection. Plasma samples were immediately frozen at -70°C until they were ready for processing. RNA was extracted by treating 200 μ l of plasma with 4 volumes of a lysis solution containing 5.75 M GuSCN, 50 mM Tris (pH 7.5), 100 mM β -mercaptoethanol, and 1 μ g of poly(rA) per ml. The poly(rA) both facilitates the precipitation of viral RNA and reduces intersample variability. The resulting lysates were incubated at 65°C for 10 min. The RNA was precipitated with 1 ml of isopropanol at room temperature, washed with 70% ethanol, and resuspended in 200 μ l of DEPC-treated water containing 400 copies of RNA control transcript (or 100 copies per 50 μ l). Samples were stored at 4°C until amplification.

RT-PCR assay. RT-PCR was carried out as a single-tube reaction with uninterrupted thermal cycling by using the GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk Conn.) as follows. A 2 \times reaction solution was prepared and dispensed in 50- μ l aliquots to thin-walled MicroAmp tubes. A 50- μ l volume of HIV-1-infected plasma lysate spiked with 100 copies of the quantitation standard was added to each tube. A dilution series consisting of 10, 20, 40, 80, and 160 copies of the quantitation standard was also amplified individually to generate a standard curve. The final reaction mixture contained the sample RNA; primers Bio-SK431 (5'-biotin-TGCTATGTCAGTTCCCCTTGGTTCTCT-3') and Bio-SK462 (5'-biotin-AGTTGGAGGACATCAAGCAGCCATGCAAAT-3') at 20 pmol each; 200 μ M dUTP; 150 μ M (each) dATP, dCTP, dGTP, and dTTP (Perkin-Elmer); 0.90 mM MnCl₂; 15% glycerol; 10 mM Tris-HCl (pH 8.3); 90 mM KCl; 2 U of UNG (Perkin-Elmer); and 10 U of *rTth* DNA polymerase (Perkin-Elmer) in a volume of 100 μ l. Prior to amplification, the reaction mixture was held for 2 min at 50°C for UNG to cleave the dUMP-containing amplified product which may have been carried over from previous reactions and to increase specificity (22). This was followed by one cycle of RT at 70°C for 15 min. Next, PCR amplification proceeded with four cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 10 s; this was followed by 24 cycles of 90°C for 10 s, 60°C for 10 s, and 72°C for 10 s. These two phases of thermal cycling improved the specificity of the reactions. Since the primers were fully complementary to the target after the initial cycles of amplification, an increase in the annealing temperature minimized nonspecific binding. Because the amplified region has a melting temperature of approximately 75°C, lowering of the denaturation temperature to 90°C sufficiently dissociated the duplex with little effect on duplexes with higher melting points. The reactions were held at 72°C for at least 10 min but for no longer than 1 h, and the reaction mixtures were then treated with an equal volume (100 μ l) of denaturation solution (Roche Molecular Systems, Branchburg, N.J.) to inactivate UNG and denature the amplified product for microwell plate analysis.

Detection of amplified product. Amplified products were detected on Amplicor microwell plates (Roche Molecular Systems) coated with specific bovine serum albumin-conjugated oligonucleotide probes: SK102 for HIV-1, SK462-SK431 *gag* system, and CP35 (5'-CATAGCACTATAGAACC TCTGCAAGCC-3') for the quantitation standard. Addition-

ally, fivefold serial dilutions of the unknown denatured products were prepared by using 1× denaturation buffer to expand the dynamic range. The undiluted denatured products of the standards and HIV-1 unknowns were analyzed on CP35 plates; the denatured unknown products were analyzed on SK102 plates. The microwell plate assay has been described previously (18). Briefly, 25 µl of the denatured product was added to 100 µl of hybridization solution in each plate well. The plates were then incubated at 37°C for 1 h with gentle mixing to allow hybridization of the biotinylated products to the respective probes. To remove unbound product, the plates were washed five times with wash buffer by using an automated plate washer (Bio-Tek Instruments, Inc., Winooski, Vt.). Following the wash, the plates were incubated with avidin-horseradish peroxidase (HRP) (Roche Molecular Systems) for 15 min at 37°C. To remove unbound conjugate, each plate was washed five more times with wash buffer. For color development, a chromogenic substrate, tetramethylbenzidine, and H₂O₂ (Roche Molecular Systems) were added to each plate, and the plates were allowed to incubate with avidin-HRP at room temperature for 10 min in the dark. The color reaction was stopped with 100 µl of stop solution, and each plate was read at 450 nm by using a microplate reader (Molecular Devices, Menlo Park, Calif.). The optical densities ranged from 0.08 to 4.0, with a linear dynamic scale ranging from 0.1 to 2.4 optical density units after subtraction of the background. Optical densities of less than 0.1 units were determined to be background signals.

A standard curve was generated by plotting the optical density against the number of input HIV-1 RNA copies. The HIV-1 copy numbers were determined by using only dilutions of the denatured product that were within the linear range of the standard curve. The copy numbers of the HIV-1 unknowns were normalized with the experimentally determined copy number of the quantitation standard by the following formula:

$$\frac{(\text{theoretical number of input copies of pNAS})}{(\text{measured number of input copies of pNAS})} \times \text{HIV-1 copy}_{\text{measured}} = \text{HIV-1 copy}_{\text{adjusted}}$$

RESULTS

Assay strategy. The four major elements of a quantitative PCR RNA assay are (i) sample preparation, (ii) RT, (iii) amplification, and (iv) detection. Each of these elements is linked, in that variation in the performance of any of these steps affects the final results. For the quantitative assay, we assessed the reproducibility of (i) a simplified RNA sample extraction protocol that employs GuSCN, (ii) RT and PCR with *rTth* DNA polymerase, and (iii) a colorimetric microwell plate assay that uses immobilized bovine serum albumin-probe conjugates.

(i) Expanding the dynamic range of the microwell assay. To facilitate the evaluation and adoption of the quantitative PCR strategy described here, we chose to use microwell plates. However, the selected microwell assays used with the HIV-1 kit (Roche Molecular Systems) provide a qualitative rather than a quantitative readout. Samples are amplified for 35 cycles, and as a result, most reactions have reached a plateau. By limiting the cycle number to 28 and detecting different amounts of the amplified product, we demonstrate that a three-log-unit dynamic range can be achieved. RNA from *in vitro*-generated transcripts of pNAS2 (10 to 3,200 copies), which served as a quantitation standard, were reverse transcribed and amplified for 28

cycles. Following amplification, twofold dilutions of the reaction products were analyzed on microwell plates containing the quantitation standard. The data from this analysis, presented in Fig. 1, suggest that a dynamic range of 10 to 400 and 400 to 3,200 input copies can be obtained with 25 and 3.25 µl of the denatured amplified product, respectively (or 12.5 and 1.56 µl of undenatured amplified product, respectively). Therefore, a dynamic range of 10 to 3,200 copies can be obtained simply by analyzing two different amounts of the amplification reaction after 28 cycles. These data indicate that the immobilized probe is limiting. Subsequent studies indicate that as many as 10⁵ input copies of HIV-1 RNA can be quantitated with additional dilutions, suggesting a four-log-unit dynamic range (data not shown).

(ii) Amplification efficiency of quantitation standard versus HIV-1. The quantitation standard should have two key characteristics. First, to simplify analysis, the amplification efficiency of the quantitation standard should be identical to that of the target. To increase the likelihood of equivalent amplification efficiency between HIV-1 and the quantitation standard template, the primer-binding regions of the quantitation standard were identical to those of the HIV-1 target and the intervening sequence was designed to be of the same size and base composition. To compare the amplification efficiency of the *gag* region primer pair SK462-SK431 (18) on the quantitation standard and HIV-1, we amplified a dilution series of an HIV-1 transcript, pCC2 (16), HIV-1 genomic RNA, and the quantitation standard transcript pNAS2 for 26, 27, and 28 cycles. Both the pCC2 transcript and HIV-1 genomic RNA were examined to ensure the validity of using pCC2 as a model system. Linear regression curves were generated for each dilution series and for each of the cycles amplified. Figure 2 shows that the optical densities and the slopes of the curves for all three targets were comparable after 26, 27, and 28 cycles, thereby demonstrating equivalent amplification of the three targets. Second, since the number of HIV-1 copies varies from sample to sample, the amplification of even a high number of copies of HIV-1 must not compromise amplification of the quantitation standard. To determine the effects of coamplification of HIV-1 on the quantitation standard, dilutions of pCC2 RNA (31 to 500 copies) were spiked with either 50, 100, or 200 copies of pNAS2 transcripts and coamplified. In addition, a dilution series of each template was amplified alone. The results of the present study indicate that the presence of one target does not interfere with amplification of the other (Table 1). We further demonstrated with clinical specimens that the presence of a greater than 100-fold excess of the HIV-1 target did not compromise amplification of the quantitation standard (data not shown).

(iii) Representative example of quantitation standard performance. Two HIV-1-seronegative plasma samples were extracted and used in spiking experiments. Each lysate was spiked with a dilution series in poly(rA) of transcripts from pCC2 and 100 copies of pNAS2. A dilution series amplified in the presence of poly(rA) alone served as a control. The amplified products were analyzed as described in Materials and Methods, and the results are summarized in Table 2. Note that the calculated copy number for both pCC2 and pNAS2 RNAs amplified in the presence of extract A were similar to those for the same templates amplified in water. When spiked into extract B, however, the calculated copy numbers following amplification and detection of both pCC2 and pNAS2 RNAs were significantly reduced. By normalizing the quantitation standard, the calculated copy numbers of pCC2 RNA amplified in the three different backgrounds

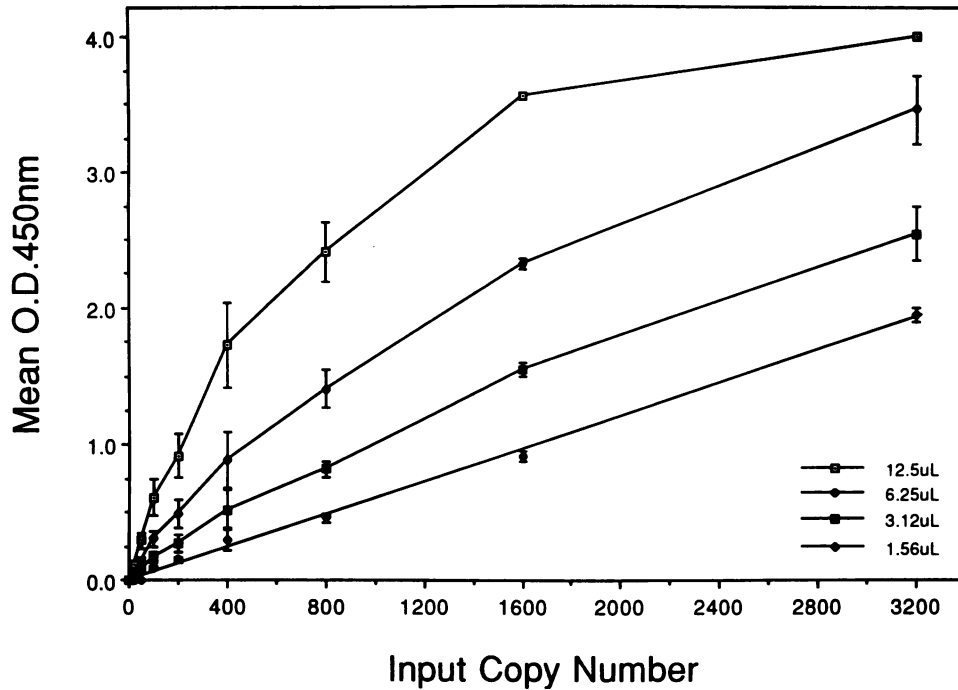


FIG. 1. Analysis of dilutions of amplified products in the microwell plate assay. A dilution series of 25 to 3,200 copies of the quantitation standard transcript pNAS2 was amplified for 28 cycles. The amplified products were denatured in an equal volume of denaturation buffer, and additional serial twofold dilutions were prepared in 1x denaturation buffer. Twenty-five microliters of each dilution, representing 12.5, 6.25, 3.12, and 1.56 μ l of the original product, was analyzed on microwell plates. The optical density (OD) at 450 nm of each dilution was plotted against the input copy number.

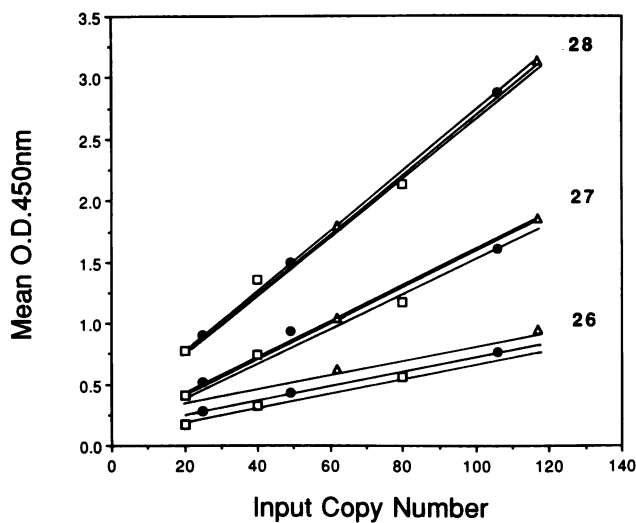


FIG. 2. Comparison of three RNA targets after 26, 27, and 28 cycles of amplification with *rTth* DNA polymerase. A dilution series (between 20 and 120 copies) of the pCC2 (HIV-1) transcript RNA (●), the pNAS2 (quantitation standard) transcript RNA (□) and HIV-1 genomic RNA (Δ) was each amplified for 26, 27, and 28 cycles and analyzed by the microwell plate assay as described in the text. The linear regression lines generated after 26, 27, and 28 cycles of amplification are represented by the lower, middle, and upper clusters of lines, respectively. OD, optical density.

closely approximated the actual input. That is, pNAS2 RNA performed as a quantitation standard.

Assay reproducibility. To assess the reproducibility of the assay, we examined each component of the assay independently, beginning with the microwell plates; this was followed by amplification and finally extraction.

(i) **Reproducibility of microwell plates.** The performance of

TABLE 1. Coamplification of various levels of pCC2 and pNAS^a

No. of input copies		Optical density		Calculated copy no.		Adjusted pCC2 copy no.
pCC2	pNAS	pCC2	pNAS	pCC2	pNAS	
500	50	2.139	0.174	459	39	596
250	50	1.356	0.230	292	51	289
125	50	0.655	0.182	142	40	177
62	50	0.431	0.245	94	54	87
31	50	0.151	0.239	34	52	32
500	100	2.172	0.556	466	120	387
250	100	1.304	0.416	280	90	310
125	100	0.705	0.505	152	109	139
62	100	0.279	0.538	61	116	53
31	100	0.154	0.404	34	88	39
500	200	2.054	0.915	441	197	448
250	200	1.084	1.100	233	237	197
125	200	1.048	0.821	226	177	255
62	200	0.384	0.780	84	168	99
31	200	0.145	0.852	32	184	35

^a A dilution series of pCC2 was amplified in the presence of either 50, 100, or 200 copies of pNAS for 28 cycles. Following analysis, the levels of pCC2 were treated as unknowns and the copy number was determined as described in the text.

TABLE 2. Coamplification of pCC2 and pNAS in different extracts^a

Sample	No. of input copies		Optical density		Unadjusted pCC2 copy no.	Calculated pNAS copy no.	Adjusted pCC2 copy no.
	pCC2	pNAS	pCC2	pNAS			
Extract A	500	100	1.490	0.325	519	124	419
	250	100	0.770	0.303	275	117	235
	125	100	0.373	0.216	141	88	161
	62	100	0.240	0.304	96	117	82
	31	100	0.088	0.315	44	121	36
	No plasma	0	0	0	0	0	0
Extract B	500	100	0.683	0.110	229	52	444
	250	100	0.345	0.091	131	45	290
	125	100	0.192	0.130	79	58	136
	62	100	0.092	0.121	46	55	83
	No plasma	0	0	0	0	0	0
Control (no plasma)	500	100	1.355	0.284	474	111	427
	250	100	0.605	0.259	219	102	215
	125	100	0.389	0.251	146	99	147
	62	100	0.183	0.287	76	112	68
	31	100	0.098	0.311	48	120	40
	0	0	0	0	0	0	0

^a A dilution series of pCC2 was spiked with 100 copies of pNAS and was coamplified in the presence of two different extracts for 28 cycles. Five levels of pNAS were also amplified independently to generate a standard curve. Following analysis on the microwell plates, pCC2 samples were treated as unknowns and the copy numbers were determined as described in the text. The optical density of the negative plasma was subtracted from each sample.

the microwell plates was evaluated by analyzing amplified products on multiple wells both within and between plates. The coefficient of variation (CV) between replicate wells on any given plate averaged less than 3.7%; an average CV of 6% was observed between plates.

(ii) **Reproducibility of replicate amplifications.** Amplifications were minimally performed in duplicate. A compilation of data from 117 replicate reactions indicated that 86 (74%) of the amplifications had CVs of $\leq 15\%$, 17 (14%) had CVs of between 15 and 20%, and 14 (12%) had CVs of between 21 and 33%. These CVs include detection of the amplified product in the microwell plates.

(iii) **Reproducibility of replicate extractions.** The reproducibilities of duplicate extractions and amplifications were evaluated on 10 samples. Of eight samples that harbored greater than 500 copies of HIV-1 per 50 μ l of plasma, seven samples had a CV of less than 12% and one sample had a CV of 31% between duplicate extractions. Since replicate amplifications of both extracts showed good reproducibility, the differences observed principally represent variations in sample extraction and/or recovery. The CVs for samples with 100 or fewer copies HIV-1 RNA are higher ($\sim 37\%$). It should be noted that just as stochastic sampling of a sample with an average of one copy per aliquot tested is only positive a fraction of the time, we expect higher CVs in samples with 10 or fewer copies because of Poisson distribution. A large sample size should circumvent this variability.

(iv) **Efficiency of viral recovery from plasma.** To determine the efficiency of viral RNA recovery, either 2, 20, or 200 μ l of acid citrate glucose-treated plasma from each of two samples was extracted. When less than 200 μ l of plasma was used, the volumes were brought up to 200 μ l with HIV-1-seronegative plasma. The extracted RNAs were all resuspended in 200 μ l of water, and 50 μ l was used for PCR. RNA extracted and amplified from 2 and 20 μ l of plasma gave signals that were comparable to those amplified from the 200- μ l input, when corrected for the dilution factor. Therefore, the efficiency of viral RNA recovery from plasma is not

affected by the virus titer even when virus is present at low levels. Preliminary results with plasma collected in heparin indicated significant inhibition of the polymerase (17); modifications in sample preparation procedures should alleviate the inhibition. Plasma collected in EDTA is suitable (data not shown).

(v) **Absence of proviral DNA sequences from plasma lysates.** Some investigators have reported the presence of HIV-1 proviral DNA sequences in the plasma of HIV-1-seropositive individuals (14). Even though those reports have not been confirmed, we wanted to ascertain whether proviral DNA was present in plasma. The presence of proviral DNA in plasma lysates would compromise quantitation of viral RNA and would require additional treatment (e.g., with DNase) of the plasma to eliminate any contaminating DNA. Fifty microliters of plasma lysates from 20 HIV-1-seropositive individuals was analyzed for the presence of proviral DNA by using *rTaq* DNA polymerase and 40 cycles of amplification to ensure detection of even minute amounts of provirus. In striking contrast to the reports of Trono (39) and Lori et al. (27), proviral DNA was not detected in any of these samples, even though about one-third of the samples harbored greater than 40,000 copies of viral RNA per ml of plasma input.

RT-PCR results for serially diluted clinical specimens. A coded panel was constructed by serially diluting fivefold an HIV-1-positive, acid citrate glucose-treated plasma sample into an HIV-1-negative, acid citrate glucose-treated plasma sample. The samples were extracted, amplified in duplicate, and analyzed as described in Materials and Methods. The panel consisted of duplicate undiluted samples and 1:5, 1:25, 1:125, and 1:625 dilutions of an HIV-1-positive sample.

The results of that analysis are given in Table 3. As evidenced by the data, each sample was correctly ranked. To assess the accuracy of the data, the copy number determined for each dilution was compared with the predicted copy number by using the number in an undiluted sample as the baseline. Results of that study demonstrated that the assay is reproducible and that fivefold differences

TABLE 3. Analysis of a coded panel of serially diluted HIV-1-infected plasma samples

Sample	Dilution	No. of HIV-1 copies/50 μ l	
		Measured	Predicted ^a
B	Undiluted	1,567	NA
H	Undiluted	1,544	NA
C	1:5	321	311
G	1:5	486	311
E	1:25	86	62
I	1:25	120	62
A	1:125	15	12
J	1:125	21	12
D	1:625	2	2
F	1:625	3	2
P	Seronegative (diluent)	Negative	0
Q	Seronegative (diluent)	Negative	0

^a The number of predicted copies was determined by dividing the average number of undiluted copies by the dilution factor. NA, not applicable.

are easily discernible. In fact, the calculated copy numbers reflected, within twofold, the predicted copy numbers. These data further support the earlier observation that RNA can be efficiently recovered from plasma, even when RNA is present at a low number of copies.

Quantitation of HIV-1 RNA during acute infection. Plasma samples from two seroconversion panels (Boston Biomedica, Inc.), one collected in 1981 (panel E) and the other collected in 1988 (panel G), were examined. The serological and antigen data (provided by the supplier), along with quantitative PCR results for these panels, are tabulated in Tables 4 (panel G) and 5 (panel E). In samples from panel G, high levels of HIV-1 RNA were detected at least 2 weeks prior to seroconversion; the levels then declined, with the viral titer being at its lowest level at the time of seroconversion. HIV-1 p24 antigen, which was detected at least 2 weeks prior to seroconversion, became undetectable after seroconversion. In contrast, HIV-1 RNA was detected both before and after seroconversion. In samples from panel E, the detection of HIV-1 RNA coincided with the detection of p24 antigen. At the time of seroconversion, however, p24 antigen was no longer detectable. Of particular note was the approximately 2-week spike in the levels of HIV-1 RNA

TABLE 4. Detection of HIV-1 in longitudinally collected plasma samples (panel G)

Specimen collection date (mo/day/yr)	Anti-HIV ELISA ^a			HIV p24 antigen ELISA		No. of HIV RNA copies/ml by RT-PCR (Roche)
	Abbott	Genetic Systems	Organon Teknika	Coulter	Abbott	
11/14/88	-	-	-	+	+	38,800
11/17/88	-	-	-	+	+	105,000
11/21/88	-	-	-	+	+	75,500
11/25/88	-	-	-	+	+	147,000
11/28/88	-	-	+	+	-	21,400
12/2/88	-	-	+	+	-	2,140
12/8/88	-	+	+	-	-	1,620
12/12/88	+	+	+	-	-	480
12/16/88	+	+	+	-	-	3,180
12/19/88	+	+	+	-	-	3,840
4/16/89	+	+	+	+	+	5,860
4/30/89	+	+	+	+	+	9,580

^a ELISA, enzyme-linked immunosorbent assay.

TABLE 5. Detection of HIV-1 in longitudinally collected plasma samples (panel E)

Specimen collection date (mo/day/yr)	Anti-HIV ELISA ^a (Abbott)	HIV p24 antigen ELISA (Pharmacia)	No. of HIV RNA copies/ml by RT-PCR (Roche)
4/14/81	-	-	ND ^b
4/21/81	-	-	ND
5/5/81	-	-	ND
5/19/81	-	-	ND
5/26/81	-	-	ND
6/2/81	-	-	ND
6/16/81	-	-	ND
7/7/81	-	+	10,800
7/14/81	-	+	111,000
8/18/81	+	-	1,200

^a ELISA, enzyme-linked immunosorbent assay.

^b ND, not detected.

which presumably corresponds to the symptoms of the acute phase of the retroviral syndrome. Consistent with other reported studies (5, 9), a significant drop in HIV-1 viral load was observed with the appearance of antibodies in samples from both panels E and G. This reduction in viremia is expected to be the result of cellular and humoral mediated immune suppression. Interestingly, the immune system is capable of substantial suppression only for a matter of weeks before the elevated level of virus associated with a persistent infection is established. Furthermore, these data demonstrate that archival clinical material collected at the inception of the AIDS epidemic can be used, even though they may not have necessarily been collected and stored under optimal conditions. The analytical sensitivity of this assay is 10 copies per 50 μ l of plasma or 200 copies per ml of plasma.

DISCUSSION

We described an amplification system that uses *rTth* DNA polymerase to catalyze both RT and PCR similar to that recently described for hepatitis C virus RNA amplification (42). Since the enzyme is thermostable and thermoactive, RT occurs at elevated temperatures, which reduces nonspecific priming events while it concomitantly facilitates amplification through G-C-rich regions with stable secondary structures. The thermoactivity of the enzyme also permits incorporation of UNG into the reactions, which eliminates carryover molecules from compromising the analysis. Enzymes that are conventionally used for RT, such as murine leukemia virus reverse transcriptase and avian myeloblastosis virus reverse transcriptase, have temperature activity profiles that are similar to those of UNG; as a consequence, the dU-containing cDNAs synthesized by these enzymes are rapidly degraded by this glycosylase. Additional measures must be taken to sequester UNG from the murine leukemia virus or avian myeloblastosis virus reverse transcriptase if a carryover prevention strategy were to be used. With *rTth* DNA polymerase, all components of the assay are added together and the reactions are carried out in a single tube.

We demonstrated that the amplifications are sensitive and reproducible. A quantitation standard similar to that described previously (41) was incorporated and successfully used in the normalization of reaction variability. Quantitation can easily be performed on microwell plates with

colorimetric detection. Our results from replicate extractions of the same sample as well as a dilution panel indicate that the assay may quantitate the viral load to within a factor of 2.

In the current assay, 200 μ l of plasma was extracted and the equivalent of 50 μ l of plasma was analyzed per amplification. The necessity of using higher plasma levels as input rests not with the analytic sensitivity of the assay but with the level of virus in the specimen. A recent report (33) indicates much higher levels of viremia than were measured previously. While only one amplification of the test sample was required, duplicate amplifications would provide added confidence in the copy number determinations. The amplified products were analyzed on two plates, one for HIV-1 and the other for the quantitation standard. To ensure that the samples fell within the dynamic range of the assay, multiple volumes of the denatured amplified reaction were assayed for HIV-1. We demonstrated that 3.25 to 25 μ l of the denatured amplified product generates linear detection from 10 to 3,200 input copies. Recent studies indicate that the colorimetric microwell detection format has at least a four-log-unit dynamic range when the described dilution methodology was incorporated (data not shown). The majority of samples are expected to harbor fewer than 100,000 copies per ml. Detection of a single level of the quantitation standard suffices.

The quantification of HIV-1 RNA in plasma by PCR may prove to be a better measure of infection status than quantification of culturable virus, for multiple reasons. First, RNAs from both infectious and noninfectious particles will be detected by PCR. Given that physical particles may be present at a 10^4 - to 10^7 -fold excess over the number of infectious particles (24), PCR is more sensitive than the *in vitro* propagation techniques. The fraction of cultivatable virus may be confounded by the length of time a virus remains in peripheral blood and inactivation by neutralizing or cross-reacting antibodies. Second, since pathogenic mechanisms have been proposed for inactive but antigenically intact viral particles, as reviewed previously (32), a measure of genomic RNA from total particles may prove to be a better marker of disease status. Third, the amount of RNA released in the plasma is an indirect measure of the transcriptional status of those infected cells that shed virus. The transcriptional activity of the virus is, in turn, associated with the expression of viral and cellular proteins that participate in pathogenesis.

The required sensitivity of a quantitative assay depends largely on the patient population. The majority of symptomatic patients with low CD4 cell counts have been shown to have higher viral titers than asymptomatic individuals with high CD4 cell counts, although a broad range of titers has been observed at all stages of infection (7, 15). The variability in virus titers in plasma among infected individuals suggests that only by following the level of viremia in plasma in a particular individual over time can the results assist in evaluating therapeutic efficacy. In order to quantitate HIV-1 RNA in patients encompassing the full spectrum of CD4 cell counts and disease states, an assay with the ability to detect as few as 200 copies per ml is required; otherwise, inordinately large volumes of plasma will be required to monitor viremia. For example, treating infected neonates with antiviral drugs is gaining acceptance; however, only relatively small volumes of plasma can be obtained from potentially infected neonates.

The quantitative ability of PCR may be influenced by the heterogeneity of the viral genome. However, since the level

of RNA in sequential samples from the same individual will be examined, the effects of sequence heterogeneity are expected to be minimal. Several strategies have been used to minimize the likelihood of differential amplification as a result of primer-template mismatches. Beyond selecting primers to highly conserved regions, primers that are at least 25 bases in length and that terminate in a T residue can better accommodate mismatches (21). It should be noted that primer-template mismatches occur only during RT and the first cycle of amplification; the primers and template are fully complementary in subsequent cycles. Mismatches between DNA primers and RNA templates are expected to be better tolerated than DNA-DNA duplexes, given the greater stability of RNA-DNA duplexes (38). Lowering the RT temperature from the 70°C used in the present study should further accommodate primer-template mismatches. In a previous study (21), we demonstrated that primer-templates with as many as five mismatches can be extended at 72°C following a 60°C annealing step. Preliminary studies, predicted by the reverse transcriptase thermal activity profile of *rTth* DNA polymerase, indicate that temperatures significantly lower than 70°C can readily be used (28a).

The quantitative HIV-1 RNA assay described here will prove to be a valuable addition to the analytical tools used to monitor viral infection. While we addressed issues involving quantitative amplification and detection, questions concerning optimal sample collection and storage conditions remain. For example, what is the stability of virions in plasma? Is a chaotropic reagent required to stabilize the viral RNA? What are the effects of differential collection, storage, and processing? Studies by Busch et al. (4) and Cuypers et al. (8) on hepatitis C virus indicate a significant reduction in HCV RNA when whole blood and serum are stored at room temperature; only a small reduction in signals was observed after storage of serum at 4°C. With virus cultivation, Pan et al. (31) found that HIV-1-infected plasma stored at -70°C for several months maintains a stable level of free virus. We are extending the sample collection and storage studies. Results of our preliminary studies indicate that, optimally, sera or plasma should be separated from cells within 3 h of collection and should be stored at -70°C. However, in the seroconversion panels examined in the present study, the results indicate that specimens collected under potentially less than optimal conditions and frozen for up to 12 years yield qualified but informative data.

The titers obtained from the seroconversion panels were up to 100-fold less than those reported by Piatak et al. (33). The differences in the observed titers are difficult to address, because potential explanations for the differences are numerous. First, as noted previously, the seroconversion panels reported here have been stored for long periods of time (5 and 12 years). Second, until identical specimens are tested in both systems, patient-to-patient differences cannot be excluded. Third, the standards used for quantitation are also unique to each system. Finally, we should note that a recent modification in our sample extraction protocol and recalibration of our standards have resulted in an increase in copies by 20- to 50-fold. In an RNA quantitation pilot study coordinated by the AIDS Clinical Trials Group, the titers that we obtained, with the noted improvements, are consistent with those obtained by multiple participating laboratories.

Considerable interest has been generated in the development of PCR assays that will detect mutations in the reverse transcriptase gene of isolates resistant to antiviral agents. The use of PCR to identify these mutations has been

complicated by (i) the increasing number of different mutations associated with drug resistance, (ii) differential resistance depending on the reverse transcriptase genotype, and (iii) different mutations for each antiviral drug (20, 23, 30, 37). A quantitative assay that measures the virus titer in plasma may provide an alternative and simpler method of monitoring the functional consequences of resistance to antiviral drugs.

The biological variability of HIV-1 is increasingly being linked to disease progression. Recently, Groenink et al. (13) reported that the conversion of nonsyncytium-inducing variants to syncytium-inducing variants preceded accelerated CD4⁺ T-cell loss and rapid progression to AIDS in 50% of the infected individuals studied. It will be of interest to determine whether the conversion of nonsyncytium-inducing variants to syncytium-inducing variants coincides with an increase in viral load.

The quantitative assay described here provides two additional benefits relative to previously reported assays. First, the greater analytical sensitivity of the assay may lead to greater diagnostic sensitivity and, therefore, may allow a larger number of infected individuals to be recruited and followed in clinical trials. Second, individuals with low viral burdens who may be more responsive to therapy can be followed with this sensitive assay. Increasingly, investigators have proposed that the modest progress in therapeutic treatment for HIV-1 infection may be due to relatively late intervention.

Ultimately, the utility of a quantitative assay for HIV-1 RNA will depend on the extent of viral titer fluctuation within an infected individual over time. The clinical utility of the assay in monitoring disease progression or responsiveness to therapy can be fully realized only if a patient's therapeutic response exceeds normal viral fluctuations. The availability of a readily usable and reliable quantitative assay for RNA provides a means of rapidly addressing this question.

Although the quantitative assay was developed to monitor HIV-1 levels in plasma, the procedure is generally applicable to other RNA targets, including other retroviruses and RNA viruses. Modifications in the procedure should further enable the quantitation of intracellular RNA (mRNA) as well as DNA.

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