JOURNAL OF CLINICAL MICROBIOLOGY, July 2008, p. 2206–2211 Vol. 46, No. 7
0095-1137/08/$08.00+0 doi:10.1128/JCM.00055-08

Highly Sensitive Methods Based on Seminested Real-Time Reverse Transcription-PCR for Quantitation of Human Immunodeficiency Virus Type 1 Unspliced and Multiply Spliced RNA and Proviral DNA▽

Alexander O. Pasternak, Karen W. Adema, Margreet Bakker, Suzanne Jurriaans, Ben Berkhout, Marion Cornelissen, and Vladimir V. Lukashov*

Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Received 11 January 2008/Returned for modification 27 February 2008/Accepted 30 April 2008

The effectiveness of highly active antiretroviral therapy (HAART), the standard of care for the treatment of human immunodeficiency virus type 1 (HIV-1) infection, is assessed by measuring the viral RNA load in plasma. A patient is considered to be successfully treated when the HIV-1 load in plasma stays below the detection limit of commercial assays. However, virus replication and evolution do continue in patients under HAART, which may eventually result in the development of drug-resistant HIV-1 strains and therapy failure. To monitor this low-level virus replication in peripheral blood mononuclear cells (PBMC), sensitive methods are required to measure HIV-1 molecular markers. We report the development of highly sensitive methods for the quantitation of unspliced and multiply spliced HIV-1 RNA and proviral DNA in PBMC. The methods are based on innovative seminested real-time reverse transcription-PCR (RT-PCR) that combines the accuracy and precision of real-time PCR and the sensitivity of nested PCR. We show that the newly developed methods are superior to the conventional single-step real-time RT-PCR in their sensitivity, accuracy, dynamic range, and the power of quantitative detection of HIV-1 RNA and DNA in clinical samples. These easy-to-perform methods can be widely used in research, including clinical studies, to monitor intracellular processes of virus replication.

Human immunodeficiency virus type 1 (HIV-1) load in blood plasma, as measured by the number of copies of HIV-1 RNA, is a major laboratory marker widely used in clinical practice. Higher virus loads are directly linked to more rapid progression to AIDS in HIV-1-infected individuals. The effectiveness of highly active antiretroviral therapy (HAART) is also assessed by measuring the HIV-1 load in plasma. A patient is considered to be successfully treated by HAART when HIV-1 load in plasma stays below the detection limit of commercial assays, which is currently 50 copies of HIV-1 RNA per ml of plasma. However, in spite of its clinical success, HAART cannot eradicate the virus, mainly due to the persistence of various viral reservoirs, including latently infected resting $CD4^+$ cells (14, 20). Recent studies demonstrated that both virus replication and evolution do continue in (some) patients even when HIV-1 RNA in plasma is undetectable, and therapy is considered to be successful (4, 11, 13, 19, 23). HAART failure, as a result of development of drug-resistant HIV-1 strains, is a common problem (5). Thus, special attention should be given to characterizing HIV-1 residual replication by studying its molecular markers in peripheral blood mononuclear cells (PBMC). In particular, the amounts of cell-associated HIV-1 RNA, both unspliced RNA (usRNA) and multiply spliced RNA (msRNA) forms and proviral DNA (prDNA) should be quantified. Of these, the expression of msRNA species that encodes Tat and Rev proteins may be linked to productive infection (21, 25), whereas the amounts of usRNA and prDNA may reflect the size of the pool of latently infected cells. However, systematic studies of the relationships between the cellular HIV-1 RNA/DNA levels and therapy outcome are hindered by the extremely low copy numbers of HIV-1 RNA/DNA in PBMC under HAART. Therefore, development of highly sensitive methods for quantitation of cellular forms of HIV-1 RNA/DNA is essential.

Real-time reverse transcription-PCR (RT-PCR) is currently the preferred method for quantitation of HIV-1 RNA/DNA in cells (7, 9). However, despite their accuracy and specificity, single-step real-time RT-PCR methods using the TaqMan detection chemistry are unable to reliably quantify <100 copies of HIV-1 RNA/DNA target per reaction in the context of total cellular RNA/DNA (8). This evokes the possibility of yielding false-negative results when PBMC material from patients under HAART is studied, especially when limited amounts of clinical material are available for analysis. Methods that use Sybr green-based detection chemistry to detect HIV-1 RNA/DNA may be more sensitive (8) but are prone to false-positive results, because DNA binding dyes do not bind in a sequence-specific manner. With a theoretical detection limit of one molecule per reaction, nested PCR is considered a more sensitive

* Corresponding author. Mailing address: Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam, Academic Medical Center, University of Amsterdam, Meibergdreef 15, Amsterdam 1105 AZ, The Netherlands. Phone: 31-20-5665861. Fax: 31-20-5669064. E-mail: v.lukashov@amc.uva.nl.

▽ Published ahead of print on 7 May 2008.

TABLE 1. Oligonucleotide primers and probes used in this study

Assay	Genomic region	Primer or probe	Type[a]	Sequence (5′–3′)	Genomic location[b]	T_m (°C)
usRNA, prDNA	*gag*	GAG1	Sense I, II	TCAGCCCAGAAGTAATACCCATGT	1280–1303	58.3
		SK431	Antisense I	TGCTATGTCAGTTCCCCTTGGTTCTCT	1474–1500	61.5
		GAG2	Antisense II	CACTGTGTTTAGCATGGTGTTT	1341–1362	55.1
		GAG3	Probe	FAM-ATTATCAGAAGGAGCCACCCCACAAGA-TAMRA	1311–1337	61.7
msRNA	*tat*/*rev*	ks1	Sense I	CTTAGGCATCTCCTATGGCAGGAA	5956–5979	59.0
		mf83	Antisense I, II	GGATCTGTCTCTGTCTCTCTCTCCACC	8433–8459	61.7
		mf84	Sense II	ACAGTCAGACTCATCAAGTTTCTCTATCAAAGCA	6012–6045	60.4
		ks2-tq	Probe	FAM-TTCCTTCGGGCCTGTCGGGTCCC-TAMRA	8399–8421	67.8

[a] I, first PCR; II, second (real-time) PCR.
[b] That is, relative to the sequence of HXB2 reference strain of HIV-1.

method than real-time PCR. However, only semiquantitative data can be produced with this method. In addition, it requires labor-intensive and time-consuming experimental procedures. In contrast, quantitative nested real-time PCR, an approach recently developed for detection and quantitation of several pathogens and tumor markers (3, 12, 16, 17, 22), allows increasing the assay sensitivity dramatically without losing the quantitative factor. Here we report the development of highly sensitive seminested real-time RT-PCR methods for the detection and quantitation of HIV-1 RNA and DNA in PBMC samples from patients under HAART, with a detection limit of four copies per reaction and a linear range of 6 orders of magnitude.

MATERIALS AND METHODS

Patients and clinical samples. In the present study we used the archival clinical samples from the patients who were participating in the Amsterdam Cohort Studies (ACS) on HIV infection and AIDS. The ACS have been conducted in accordance with the ethical principles set out in the declaration of Helsinki, and written informed consent is obtained prior to sample collection. The study was approved by the ACS medical committee.

Cells and nucleic acid extraction. PBMC were isolated by standard Ficoll-Hypaque density gradient centrifugation and frozen in aliquots in liquid nitrogen. Total cellular nucleic acids were extracted from PBMC samples (0.5 to 3 million PBMC was used for one extraction) according to the isolation method of Boom et al. (1), eluted in water, and frozen in aliquots at −80°C until further processing.

Seminested real-time PCR assays. For seminested real-time PCR for HIV-1 prDNA, the eluted cellular DNA, or serial dilutions of plasmid DNA standards, were directly subjected to two rounds of PCR amplification. The primer pair used in the first PCR, GAG1 and SK431, amplifies a region within the HIV-1 *gag* gene (Table 1). The first round of the PCR was performed on a conventional PCR machine (GeneAmp PCR System 9700; Applied Biosystems) in 25 μl of PCR mix containing 5 μl of template, 20 mM Tris (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.4 mM concentrations of deoxynucleoside triphosphates, 1 U of AmpliTaq (Applied Biosystems), and 50 ng each of both primers. The PCR settings were as follows: 94°C for 3 min, followed by 15 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The product of the first PCR was subsequently used as a template in the second, seminested, real-time, PCR amplification, performed on the ABI Prism 7000 real-time PCR machine (Applied Biosystems) using TaqMan detection chemistry. A total of 2 μl of the first PCR product was diluted to 50 μl with PCR mix containing 25 μl of 2*Pt qPCR mix (Invitrogen), 1 μl of ROX reference dye (Invitrogen), 1 mM $MgCl_2$, 0.2 μM concentrations of each of both primers, GAG1 and GAG2, and 0.2 μM TaqMan dual-labeled fluorescent probe GAG3. Real-time PCR settings were as follows: 50°C for 2 min, then 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. The amplicon sizes were 221 bp for the first PCR and 83 bp for the second (real-time) PCR.

Seminested real-time PCR on HIV-1 RNA were performed as follows: the eluted cellular RNA, or serial dilutions of synthetic RNA standards, were first subjected to DNase treatment, to remove HIV-1 prDNA which could interfere with the quantitation, and subsequently to RT. DNase treatment was performed with DNase I (Invitrogen) for 15 min at room temperature, after which DNase was heat inactivated for 10 min at 70°C in the presence of EDTA and dithiothreitol. RT was performed with random hexamers as primers and SuperScript III (Invitrogen) at 42°C for 60 min according to the manufacturer's instructions, after which reverse transcriptase was heat inactivated for 10 min at 70°C. cDNA was divided into two portions, one of which was used in the usRNA assay, and the other was used in the msRNA assay. Two rounds of PCR were performed under the same PCR conditions as described above for the prDNA assay. For the usRNA assay, real-time PCR was run for 45 cycles, and for the msRNA assay, real-time PCR was run for 50 cycles. For the usRNA assay, the same primers and fluorescent probe were used as for the prDNA assay. The first PCR of the msRNA assay was performed with the primer pair ks1 and mf83, which amplifies the msRNA species encoding the Tat and Rev proteins (Table 1). Subsequently, the seminested real-time PCR of the msRNA assay was performed with the primers mf83 and mf84 and the TaqMan fluorescent probe ks2-tq. The amplicon sizes were 171 bp for the first PCR and 115 bp for the second (real-time) PCR of the msRNA assay.

Single-step real-time PCR (for DNA and RNA assays) was performed as described above, with the omission of the first PCR.

Standard curves and normalization. The amounts of PBMC-derived HIV-1 DNA and RNA were determined by using the standard curve method. The standard curves were built based on the results of seminested real-time (RT-)PCR, run in parallel, in which serial dilutions of the external standards were used. As external standards, we used plasmid DNA or synthetic RNA molecules, corresponding to the regions of interest. Specifically, for the prDNA assay, we used the plasmid pLAIΔRT, which is a molecular clone of HIV-1 harboring a deletion of the RT gene (6). The external standards for the RNA assays were synthetic runoff transcripts generated from the linearized plasmids pGAG2-A5 (for the usRNA assay) and pEX4+7 (for the msRNA assay). The pGAG2-A5 plasmid contains HIV-1 *gag* sequences, and pEX4+7 represents a partial sequence (exons 4 and 7) of HIV-1 msRNA encoding the Tat and Rev proteins. The lengths of the synthetic transcripts were 955 nucleotides (nt) for pGAG2-A5 and 860 nt for pEX4+7. The concentrations of DNA and RNA standards were determined spectrophotometrically and recalculated to copies/μl. Master stocks of the standards, diluted to 10^{11} copies/μl for RNA standards and to 10^9 copies/μl for DNA standards, were frozen in aliquots at −80°C until use.

The amounts of PBMC-derived HIV-1 DNA and RNA were normalized to the total cellular input in the first PCR, which was quantified in separate real-time PCRs by using a beta-actin detection kit (Applied Biosystems). To control the integrity of the RNA assays, we used the rRNA detection kit (Applied Biosystems). In addition, to assess the reproducibility of the nucleic acid extraction and RT protocols, we used an internal control. Equal amounts of the RNA-containing lysate of Phocine distemper virus were spiked into the PBMC samples (which differed in their cellular content by 2 orders of magnitude) before nucleic acid extraction. In the real-time PCR assay for Phocine distemper virus, done on this PBMC-derived material as described elsewhere (24), the mean cycle threshold (C_T) value ± the standard deviation was found to be 33.48 ± 1.05 cycles, indicating the reproducibility of nucleic acid extraction and RT-PCR/real-time PCR protocols.

Statistical analyses of quantitative data were performed by using Microsoft Excel software. The statistical tests used are specified in Results.

RESULTS

Design and validation of the seminested real-time PCR methods. Since the sensitivity of single-step real-time (RT-)PCR methods was suboptimal for the quantitative detection of HIV-1 RNA and DNA in PBMC from patients undergoing HAART (Fig. 1A), we set out to develop and validate seminested real-time RT-PCR methods. To achieve this, for the HIV-1 prDNA and usRNA assays we designed oligonucleotide primers (GAG1 and GAG2) and a dual-labeled TaqMan fluorescent probe (GAG3). The primers and probe, corresponding to the conserved region encoding Gag protein, were designed based on the sequences of different HIV-1 (subtype B) strains from the Los Alamos HIV database. As a reverse primer for the first PCR, we used primer SK431 (15). For the msRNA assay, we utilized the primers and TaqMan fluorescent probe that were initially designed by Fischer et al. (9) for their single-step real-time PCR assays. Table 1 presents the localization of the primers and probes in the HIV-1 genome.

We started the validation of the methods by testing their sensitivity and accuracy using serial dilutions of synthetic RNA/DNA standards that corresponded to the amplified regions. First, we tested the methods with different numbers of cycles of the first PCR and found that, in order to prevent nonlinear amplification, the number of cycles in the first PCR had to be limited to 15 (data not shown). Accordingly, all subsequent experiments were done with 15 cycles of the first PCR.

Next, we used the serial dilutions of standards to compare seminested and single-step real-time PCR methods for all three assays: HIV-1 usRNA, msRNA, and prDNA. Experiments were done in triplicate, separated in time by at least 1 week. In every experiment a separate master stock was used to make serial dilutions of the standards. Figure 1A shows that, for all three assays, four copies of an RNA or DNA standard per reaction could be detected by the seminested real-time PCR compared to the detection limit of 100 copies per reaction of the corresponding single-step real-time PCR. For all assays, no positive signals have been obtained from the negative controls of the first PCR, which were routinely included in the experiments, as well as from further dilutions of the standards (data not shown). Comparisons of the amplification graphs of seminested and single-step real-time PCRs (Fig. 1A, solid versus dotted lines) demonstrated that the first PCR preamplified the DNA or cDNA signals more than by a factor of 1,000. Figure 1B shows that this preamplification did not interfere with the linearity of the standard curves throughout the whole range of serial dilutions ($R^2 > 0.99$ for all three assays). On the contrary, the linearity was even slightly higher in the seminested assays. For all three assays, the linear relationship between the copy number of input RNA or DNA and the threshold cycle number (C_T value) of the seminested real-time PCR was held within the range of 10^0 to 10^6 in the semilog plot (Fig. 1B). In other words, the amounts of HIV-1 RNA or DNA could be accurately measured within the quantitation range of 6 orders of magnitude. Table 2 shows the C_T values of serially diluted standards for all three assays. The reproducibility of the C_T values of standards in the seminested assays was higher than in the respective single-step assays: the standard deviations of the C_T values were 0.43 ± 0.32 cycles (seminested) versus 0.65 ± 0.38 cycles (single step) for the usRNA assay, 0.91 ± 0.32 cycles (seminested) versus 1.05 ± 0.71 cycles (single step) for the msRNA assay, and 0.58 ± 0.44 cycles (seminested) versus 0.74 ± 0.57 cycles (single step) for the prDNA assay (means ± the standard deviations).

Detection and quantitation of HIV-1 DNA and RNA in clinical samples. We proceeded with the validation of the developed methods to assess their power of detection and quantitation of HIV-1 RNA and DNA in clinical samples. First, we validated the usRNA assay by using blood plasma samples from HIV-infected patients ($n = 18$) with plasma HIV-1 RNA loads previously measured by a commercial assay (Amplicor). The experiment has been conducted as a blind study. The mean difference between the set of values generated by the seminested real-time RT-PCR and the control set (Amplicor) was 0.31 $\log_{10}$ (standard deviation = 0.74 $\log_{10}$, 95% confidence interval = −0.03 to 0.65 $\log_{10}$), which is the acceptable difference between the values measured by two different assays, taking into account that the accepted threshold of clinically significant variability of an assay is 0.5 $\log_{10}$.

Next, we selected 14 archival PBMC samples from HIV-1 (subtype B)-infected patients receiving HAART. Samples were selected randomly with respect to the plasma HIV-1 RNA load or $CD4^+$ count. RNA and DNA were extracted from these samples and examined by using the seminested real-time PCR and, in parallel, the corresponding single-step real-time PCR methods. Samples were quantified by the standard curve method using serial dilutions of synthetic RNA and DNA standards. A sample was considered positive if its quantified amount was described above the detection limit of an assay (4 copies per reaction for the seminested methods and 100 copies per reaction for the single-step methods [see above]). Experiments were done in duplicate, and a sample was considered detected by an assay only if it was positive in both of them. The results of the quantitation, expressed as the number of copies in real-time PCR, are shown in Table 3. For all three assays, seminested real-time (RT-)PCR was clearly superior to the single-step real-time (RT-)PCR in both the power of detection and the accuracy: with the seminested methods, HIV RNA and DNA could be detected and quantified in higher proportions of clinical samples (usRNA, $P < 0.01$; msRNA, $P = 0.08$; prDNA, $P < 0.001$ [Fisher exact test]), and the coefficients of variation of the seminested methods were lower ($P < 0.001$ for all three assays [Student t test]) than those of the single-step methods.

Finally, we selected 35 additional archival PBMC samples from HIV-1 (subtype B)-infected patients under HAART, whose HIV-1 plasma RNA loads were below the detection limit of commercial assays. By the seminested real-time PCR methods, HIV-1 usRNA and prDNA were detected in 31 (89%) and 33 (94%) samples, respectively. On the other hand, msRNA was detectable in six samples (17%), probably reflecting the cessation of viral transcription under HAART.

DISCUSSION

Having combined the accuracy and precision of real-time PCR and the sensitivity of nested PCR, we developed and validated new, highly sensitive methods for quantitation of HIV-1 unspliced and multiply spliced RNA and proviral DNA.

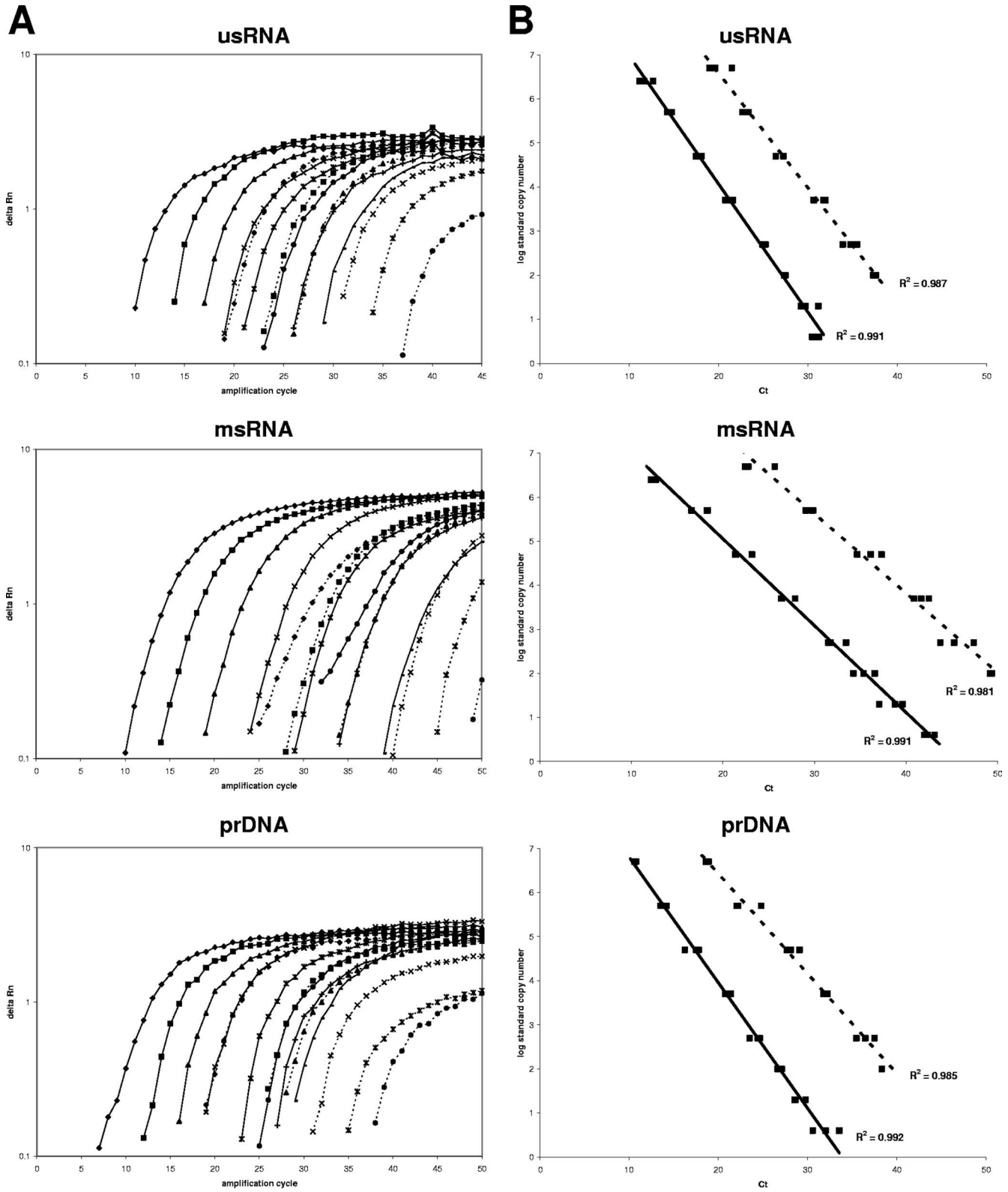

FIG. 1. Comparison between seminested (solid lines) and single-step (dotted lines) real-time PCR methods. (A) Comparison of individual amplification graphs. Signals were obtained from amplification of serially diluted synthetic RNA or DNA standards: ◆, 2.5×10^6 copies (usRNA and msRNA, seminested methods) or 5×10^6 copies (prDNA, seminested method and all assays, single-step methods); ■, 5×10^5 copies; ▲, $5*10^4$ copies; ×, 5×10^3 copies; ✳, 5×10^2 copies; ●, 1×10^2 copies; crosses, 20 copies; -, 4 copies. (B) Comparison of standard curves. The starting copy number of each serially diluted synthetic RNA or DNA standard was plotted against the corresponding threshold cycle number on a semi-log scale and fitted with a linear regression model.

TABLE 2. Comparison of seminested and single-step real-time PCR methods: amplification of the synthetic standards

Copy no. of standard	Mean C_T ± SD[a]					
	usRNA		msRNA		prDNA	
	Seminested real-time PCR	Single-step real-time PCR	Seminested real-time PCR	Single-step real-time PCR	Seminested real-time PCR	Single-step real-time PCR
5×10^6		20.00 ± 1.30		23.61 ± 1.78	10.66 ± 0.08	18.73 ± 0.16
2.5×10^6	11.81 ± 0.78		12.33 ± 0.30			
5×10^5	14.47 ± 0.25	22.95 ± 0.36	17.73 ± 1.01	29.54 ± 0.43	13.78 ± 0.38	23.03 ± 1.54
5×10^4	17.72 ± 0.33	26.96 ± 0.53	21.95 ± 1.05	36.03 ± 1.32	17.18 ± 0.82	28.29 ± 0.74
5×10^3	21.20 ± 0.42	31.43 ± 0.69	26.90 ± 0.86	41.66 ± 0.81	21.01 ± 0.33	31.93 ± 0.25
5×10^2	25.05 ± 0.16	34.71 ± 0.81	32.24 ± 1.05	45.47 ± 1.82	24.19 ± 0.62	36.48 ± 1.03
1×10^2	27.40 ± 0.06	37.47 ± 0.23	35.41 ± 1.16	49.27 ± 0.12	26.82 ± 0.24	38.31
2×10^1	30.02 ± 1.01	ND	38.49 ± 1.29	ND	28.99 ± 0.67	ND
4×10^0	30.78 ± 0.40	ND	42.50 ± 0.58	ND	32.05 ± 1.48	ND

[a] ND, not detected.

We have shown that the seminested real-time (RT-)PCR is superior to the conventional single-step real-time (RT-)PCR in the detection and quantitation of HIV-1 RNA and DNA and that it can be used to study HIV-1 gene expression in PBMC samples from patients receiving HAART, including samples from patients with an undetectable HIV-1 load in plasma. To our knowledge, this is the first report of a nested real-time RT-PCR method for HIV-1 RNA detection. Several groups have recently reported development of nested real-time Alu-HIV PCR on HIV-1 prDNA for analysis of HIV-1 integration sites (2, 26). Our prDNA assay is not aimed at discriminating between integrated and unintegrated forms of HIV-1. Although it is unclear at the moment whether unintegrated HIV-1 plays a functional role in patients receiving HAART (18), our method can be modified to specifically detect integrated/unintegrated HIV-1 DNA forms. Similarly, the usRNA assay can be modified for the specific detection of intracellular and extracellular RNA forms, for example, using the freeze-thaw nuclease digestion method developed by Fischer et al. (10).

Our initial attempts to analyze viral gene expression in PBMC from patients under HAART were hindered by the difficulties in obtaining quantitative data when HIV-1 RNA and DNA were present in very low copy numbers in cells, and limited amounts of starting clinical material were available for analysis. On the other hand, the detection limit of four copies of RNA or DNA per reaction of our new methods means that high amounts of starting material are no longer necessary to produce quantitative data without the risk of obtaining false-negative results. The possibility to easily study HIV-1 gene expression in PBMC becomes critical when the viral RNA load in plasma under therapy falls below the detection limit of clinical assays, making cells the only source of information about viral persistence and gene expression. Latently infected cells in peripheral blood are considered to be an important HIV-1 reservoir in patients under HAART, and the failure of HAART and the development of drug-resistant strains in a proportion of patients is most probably the result of virus replication taking place during therapy. The latter process should ideally be monitored in the clinic, provided that fast and

TABLE 3. Quantitation of HIV-1 RNA and DNA in PBMC samples by seminested and single-step real-time PCR methods

Sample code	Mean copy no. in reaction ± SD[a]					
	usRNA		msRNA		prDNA	
	Seminested real-time PCR (n = 8)	Single-step real-time PCR (n = 1)	Seminested real-time PCR (n = 5)	Single-step real-time PCR (n = 1)	Seminested real-time PCR (n = 12)	Single-step real-time PCR (n = 2)
1	ND	ND	11.96 ± 0.35	ND	ND	ND
2	ND	ND	ND	ND	8.68 ± 1.34	ND
3	10.89 ± 0.13	ND	9.44 ± 0.16	ND	10.46 ± 1.87	ND
4	19.76 ± 14.17	ND	12.11 ± 0.55	ND	8.21 ± 1.00	ND
5	6.58 ± 2.50	ND	ND	ND	6.23 ± 0.33	ND
6	ND	ND	ND	ND	ND	ND
7	86.63 ± 5.87	ND	ND	ND	49.41 ± 14.42	ND
8	ND	ND	ND	ND	29.89 ± 4.99	ND
9	197.58 ± 38.27	ND	ND	ND	45.41 ± 0.91	ND
10	21.44 ± 0.31	ND	ND	ND	10.66 ± 5.18	ND
11	ND	ND	ND	ND	7.37 ± 0.89	ND
12	30.26 ± 14.32	ND	24.65 ± 2.09	ND	16.33 ± 0.05	ND
13	ND	ND	ND	ND	84.86 ± 0.70	2,547.61 ± 2,292.91
14	859.74 ± 110.51	1,789.60 ± 1,523.18	12.84 ± 1.18	156.90 ± 28.69	156.45 ± 12.06	3,734.30 ± 3,424.77

[a] n, Number of samples detected. ND, not detected. The mean percent coefficients of variation (95% confidence intervals [where applicable]) were as follows: usRNA seminested real-time PCR, 25% (7% to 43%); usRNA single-step real-time PCR, 85%; msRNA seminested real-time PCR, 5% (2% to 8%); msRNA single-step real-time PCR, 18%; and prDNA seminested real-time PCR, 14% (6% to 22%); prDNA single-step real-time PCR, 91% (89% to 93%).

sensitive methods are available to do so. In this light, the detection and quantitation of viral DNA and RNA in PBMC of HAART-treated patients by our ultrasensitive methods is of significant clinical importance. With high sensitivity (four copies per reaction), wide linear range (6 $\log_{10}$), and relative ease in performance, our methods can be widely used in clinical research to monitor intracellular viral processes in patients treated with antiretroviral drugs. Likewise, the methods can be used in basic and clinical research to monitor the effects of various inhibitors of HIV-1. Studies are ongoing in our laboratory to apply the methods described here to study the relationships between intra-PBMC HIV-1 RNA/DNA levels and the outcome of HAART.

ACKNOWLEDGMENTS

We are grateful to Joke Brouwer for preparing the clinical samples for analysis.

This study was performed as part of the ACS on HIV infection and AIDS, a collaboration between the Amsterdam Health Service, the Academic Medical Centre of the University of Amsterdam, Sanquin Blood Supply Foundation, and the University Medical Centre Utrecht (http://www.amsterdamcohortstudies.org/). The ACS are part of The Netherlands HIV Monitoring Foundation and financially supported by The Netherlands National Institute for Public Health and the Environment. We are greatly indebted to all cohort participants for their continuous participation. The study was financially supported by the Dutch AIDS Fonds grant 2004045.

REFERENCES

1. **Boom, R., C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
2. **Brussel, A., and P. Sonigo.** 2003. Analysis of early human immunodeficiency virus type 1 DNA synthesis by use of a new sensitive assay for quantifying integrated provirus. J. Virol. **77:**10119–10124.
3. **Carrière, M., V. Pène, A. Breiman, F. Conti, S. Chouzenoux, E. Meurs, M. Andrieu, P. Jaffray, L. Grira, O. Soubrane, P. Sogni, Y. Calmus, S. Chaussade, A. R. Rosenberg, and P. Podevin.** 2007. A novel, sensitive, and specific RT-PCR technique for quantitation of hepatitis C virus replication. J. Med. Virol. **79:**155–160.
4. **Chun, T. W., D. C. Nickle, J. S. Justement, D. Large, A. Semerjian, M. E. Curlin, M. A. O'Shea, C. W. Hallahan, M. Daucher, D. J. Ward, S. Moir, J. I. Mullins, C. Kovacs, and A. S. Fauci.** 2005. HIV-infected individuals receiving effective antiviral therapy for extended periods of time continually replenish their viral reservoir. J. Clin. Investig. **115:**3250–3255.
5. **del Rio, C.** 2006. Current concepts in antiretroviral therapy failure. Top. HIV Med. **14:**102–106.
6. **de Ronde, A., M. van Dooren, L. van Der Hoek, D. Bouwhuis, E. de Rooij, B. van Gemen, R. de Boer, and J. Goudsmit.** 2001. Establishment of new transmissible and drug-sensitive human immunodeficiency virus type 1 wild types due to transmission of nucleoside analogue-resistant virus. J. Virol. **75:**595–602.
7. **Douek, D. C., J. M. Brenchley, M. R. Betts, D. R. Ambrozak, B. J. Hill, Y. Okamoto, J. P. Casazza, J. Kuruppu, K. Kunstman, S. Wolinsky, Z. Grossman, M. Dybul, A. Oxenius, D. A. Price, M. Connors, and R. A. Koup.** 2002. HIV preferentially infects HIV-specific $CD4^+$ T cells. Nature **417:**95–98.
8. **Espy, M. J., J. R. Uhl, L. M. Sloan, S. P. Buckwalter, M. F. Jones, E. A. Vetter, J. D. C. Yao, N. L. Wengenack, J. E. Rosenblatt, F. R. Cockerill III, and T. F. Smith.** 2006. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin. Microbiol. Rev. **19:**165–256.
9. **Fischer, M., B. Joos, B. Hirschel, G. Bleiber, R. Weber, H. F. Günthard, et al.** 2004. Cellular viral rebound after cessation of potent antiretroviral therapy predicted by levels of multiply spliced HIV-1 RNA encoding *nef*. J. Infect. Dis. **190:**1979–1988.
10. **Fischer, M., J. K. Wong, D. Russenberger, B. Joos, M. Opravil, B. Hirschel, A. Trkola, H. Kuster, R. Weber, H. F. Günthard, et al.** 2002. Residual cell-associated unspliced HIV-1 RNA in peripheral blood of patients on potent antiretroviral therapy represents intracellular transcripts. Antivir. Ther. **7:**91–103.
11. **Frenkel, L. M., Y. Wang, G. H. Learn, J. L. McKernan, G. M. Ellis, K. M. Mohan, S. E. Holte, S. M. De Vange, D. M. Pawluk, A. J. Melvin, P. F. Lewis, L. M. Heath, I. A. Beck, M. Mahalanabis, W. E. Naugler, N. H. Tobin, and J. I. Mullins.** 2003. Multiple viral genetic analyses detect low-level human immunodeficiency virus type 1 replication during effective highly active antiretroviral therapy. J. Virol. **77:**5721–5730.
12. **Halliday, C., Q. X. Wu, G. James, and T. Sorrell.** 2005. Development of a nested qualitative real-time PCR assay to detect *Aspergillus* species DNA in clinical specimens. J. Clin. Microbiol. **43:**5366–5368.
13. **Havlir, D. V., M. C. Strain, M. Clerici, C. Ignacio, D. Trabattoni, P. Ferrante, and J. K. Wong.** 2003. Productive infection maintains a dynamic steady state of residual viremia in human immunodeficiency virus type 1-infected persons treated with suppressive antiretroviral therapy for five years. J. Virol. **77:**11212–11219.
14. **Hermankova, M., J. D. Siliciano, Y. Zhou, D. Monie, K. Chadwick, J. B. Margolick, T. C. Quinn, and R. F. Siliciano.** 2003. Analysis of human immunodeficiency virus type 1 gene expression in latently infected resting $CD4^+$ T lymphocytes in vivo. J. Virol. **77:**7383–7392.
15. **Jackson, J. B., C. Ndugwa, F. Mmiro, P. Kataaha, L. Guay, E. A. Dragon, J. Goldfarb, and K. Olness.** 1991. Non-isotopic polymerase chain reaction methods for the detection of HIV-1 in Ugandan mothers and infants. AIDS **5:**1463–1467.
16. **Jiang, S. S., T. C. Chen, J. Y. Yang, C. A. Hsiung, I. J. Su, Y. L. Liu, P. C. Chen, and J. L. Juang.** 2004. Sensitive and quantitative detection of severe acute respiratory syndrome coronavirus infection by real-time nested polymerase chain reaction. Clin. Infect. Dis. **38:**293–296.
17. **Max, N., K. Wolf, E. Thiel, and U. Keilholz.** 2002. Quantitative nested real-time RT-PCR specific for tyrosinase transcripts to quantitate minimal residual disease. Clin. Chim. Acta **317:**39–46.
18. **Petitjean, G., Y. Al Tabaa, E. Tuaillon, C. Mettling, V. Baillat, J. Reynes, M. Segondy, and J. Vendrell.** 2007. Unintegrated HIV-1 provides an inducible and functional reservoir in untreated and highly active antiretroviral therapy-treated patients. Retrovirology **4:**60.
19. **Ramratnam, B., R. Ribeiro, T. He, C. Chung, V. Simon, J. Vanderhoeven, A. Hurley, L. Zhang, A. S. Perelson, D. D. Ho, and M. Markowitz.** 2004. Intensification of antiretroviral therapy accelerates the decay of the HIV-1 latent reservoir and decreases, but does not eliminate, ongoing virus replication. J. Acquir. Immune Defic. Syndr. **35:**33–37.
20. **Siliciano, J. D., J. Kajdas, D. Finzi, T. C. Quinn, K. Chadwick, J. B. Margolick, C. Kovacs, S. J. Gange, and R. F. Siliciano.** 2003. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting $CD4^+$ T cells. Nat. Med. **9:**727–728.
21. **Sonza, S., H. P. Mutimer, K. O'Brien, P. Ellery, J. L. Howard, J. H. Axelrod, N. J. Deacon, S. M. Crowe, and D. F. Purcell.** 2002. Selectively reduced *tat* mRNA heralds the decline in productive human immunodeficiency virus type 1 infection in monocyte-derived macrophages. J. Virol. **76:**12611–12621.
22. **Takahashi, T., and T. Nakayama.** 2006. Novel technique of quantitative nested real-time PCR assay for *Mycobacterium tuberculosis* DNA. J. Clin. Microbiol. **44:**1029–1039.
23. **Tobin, N. H., G. H. Learn, S. E. Holte, Y. Wang, A. J. Melvin, J. L. McKernan, D. M. Pawluk, K. M. Mohan, P. F. Lewis, J. I. Mullins, and L. M. Frenkel.** 2005. Evidence that low-level viremias during effective highly active antiretroviral therapy result from two processes: expression of archival virus and replication of virus. J. Virol. **279:**9625–9634.
24. **van Doornum, G. J., M. Schutten, J. Voermans, G. J. Guldemeester, and H. G. Niesters.** 2007. Development and implementation of real-time nucleic acid amplification for the detection of enterovirus infections in comparison to rapid culture of various clinical specimens. J. Med. Virol. **79:**1868–1876.
25. **Vesanen, M., M. Markowitz, Y. Cao, D. D. Ho, and K. Saksela.** 1997. Human immunodeficiency virus type1 mRNA splicing pattern in infected persons is determined by the proportion of newly infected cells. Virology **236:**104–109.
26. **Yamamoto, N., C. Tanaka, Y. Wu, M. O. Chang, Y. Inagaki, Y. Saito, T. Naito, H. Ogasawara, I. Sekigawa, and Y. Hayashida.** 2006. Analysis of human immunodeficiency virus type 1 integration by using a specific, sensitive and quantitative assay based on real-time polymerase chain reaction. Virus Genes **32:**105–113.