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Quantification of Human Immunodeficiency Virus Type 1 Proviral DNA by Using TaqMan Technology

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A protocol for quantification of human immunodeficiency virus type 1 (HIV-1) proviral DNA with the TaqMan technology was developed and validated. The assay was specific for HIV-1, with an analytic sensitivity of 10 copies and a linear dynamic range of >6 logs. Viral RNA levels, when at a stable state, were highly correlated with proviral DNA levels in 80 specimens of 18 HIV-infected children.

The use of highly active antiretroviral therapy (HAART) in human immunodeficiency virus (HIV)-infected patients can often significantly reduce the levels of viral RNA to undetectable levels in plasma in both adults and children (13–17). However, it is less clear how changes in proviral DNA levels respond to HAART in infected patients or what relative changes in proviral DNA levels precede changes in viral RNA levels after HAART induction. One of the current challenges is detection of low levels of proviral DNA in latently infected $CD4^+$ lymphocytes and other reservoirs, which can replenish and revive viral infection upon activation (3–5). Thus, a highly reproducible and accurate assay for quantification of proviral DNA would enable more in-depth evaluations of the efficacies of antiviral therapies.

Several assays for the quantification of HIV type 1 (HIV-1) proviral DNA have previously been reported, and all of these were based on the principle of conventional PCR (1, 2, 6, 10, 11). The potential limitation associated with the traditional quantitative PCR is that the DNA copy numbers are calculated on the basis of the quantities of the final amplified gene products. Since DNA is amplified exponentially during PCR, a small variation in amplification efficiency early in the thermocycling process could potentially lead to large variations in the final quantities of amplified products.

The real-time PCR, which is also known as the TaqMan or the 5′ exonuclease assay, quantifies PCR products cycle by cycle (in real time) as they accumulate (7–9). There are several advantages of using real-time PCR. (i) It does not rely on the final product of PCR amplification for quantification. DNA copy numbers are determined on the basis of the threshold cycle number (C_T), which is directly proportional to the initial copy number. (ii) It is relatively specific. If the probe binds nonspecifically to some sequences other than the target sequence, it will not be cleaved or detected as part of the amplification. (iii) It allows a wide dynamic range of detection since the measured DNA copy number is directly proportional to the initial copy number. (iv) All real-time PCR tests are performed in 96-well and closed-tube formats, allowing high-throughput testing and a greatly reduced chance of cross-contamination.

In this report, a new protocol for quantification of HIV-1 proviral DNA with the TaqMan technology is described and validated. By using this method, HIV-1 proviral DNA levels were compared in parallel with the HIV-1 RNA levels at a stable state during HAART.

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In order to obtain absolute quantification of HIV-1 proviral DNA, we constructed an internal quantification standard (QS) with known copy numbers. Four criteria were used to design the QS. (i) The nucleotide sequence of the QS probe [5′-(FAM)-TAACCCACTCGTGCACCCAACTGATCTT-(TAMRA)-3′, where FAM is 6-carboxyfluoroscein and TAMRA is 6-carboxytetramethylrhodamine] is different from the *gag* probe sequence [5′-(FAM)-ACCATCAATGAGGAAGCTGCAGAATGGG-(TAMRA)-3′], so that cross-hybridization between the QS probe and the *gag* gene target does not occur, or vice versa, when they are amplified in the same tube. (ii) The same primers (primers SK452 and SK431) are used for both the QS and the *gag* sequences. This ensures identical amplification conditions when the PCR is initiated. (iii) The GCs contents (in percent) in both the *gag* and the QS DNA targets are identical. (iv) The QS amplicon is the same length (130 bp) as the *gag* amplicon. The resulting plasmid carrying QS was designated pZErO2-QS. The effective copy number of the QS was calculated on the basis of its molecular weight and was further calibrated with a PCR copy number panel provided by the AIDS Research and Reagent Reference Program of the National Institutes of Health (NIH) (12).

The linear dynamic range and the intra- and interassay variabilities of detection of the QS and the PCR-amplified HIV-1 *gag* gene were determined by preparing 10-fold serial dilutions with copy numbers ranging from 1.0×10^1 to 1.0×10^{10} copies/reaction mixture. The real-time PCR experiments were run in triplicate on an ABI 7700 Prism sequence detector and were performed three separate times. Each datum point on the

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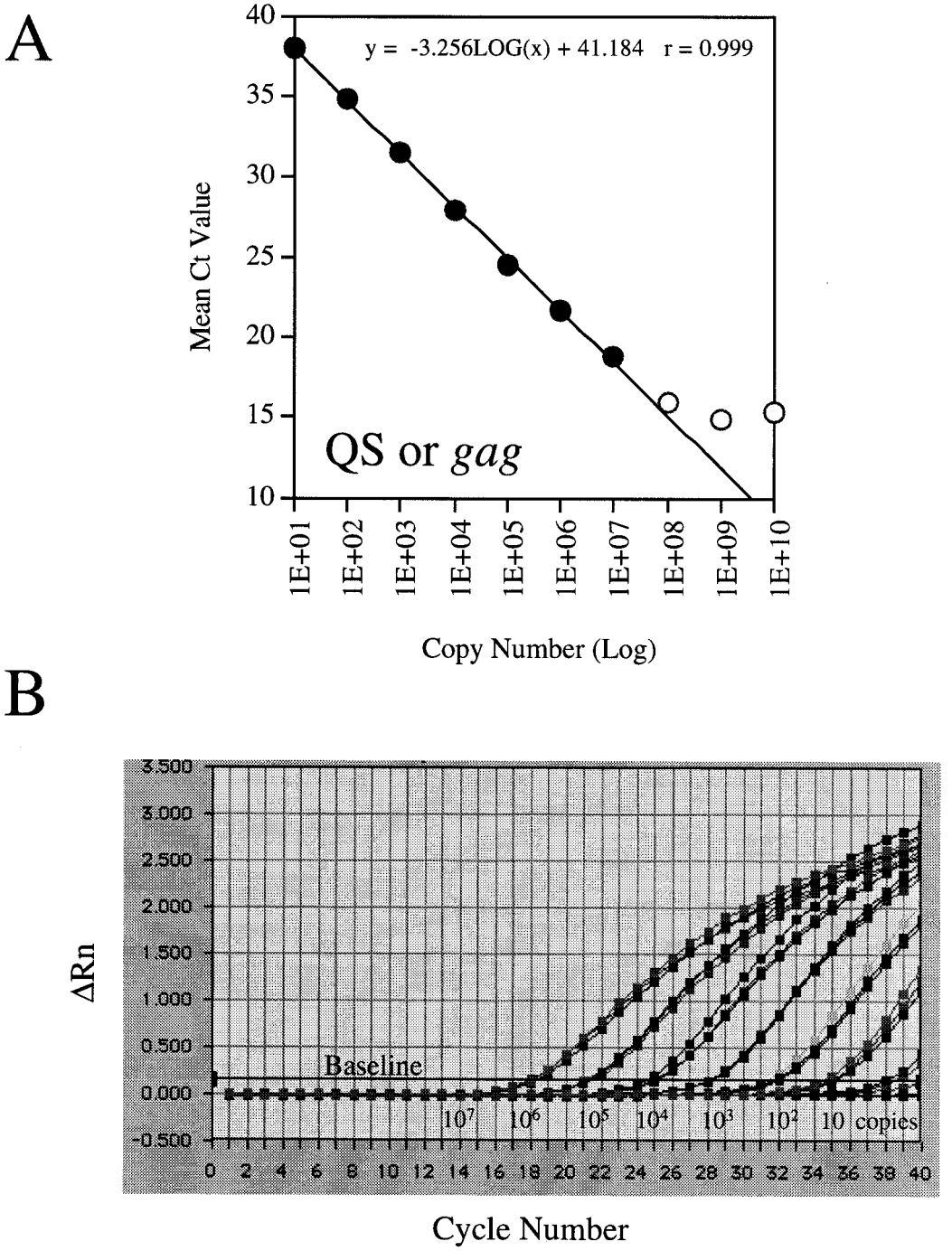

FIG. 1. Linear dynamic range and reproducibility as quantified by QS or the *gag* gene. (A) The mean C_T plotted against the QS or the *gag* gene copy number yields a line which is linear over more than 6 logs (closed circles). Open circles represent the nonlinear portion of the curve. Each datum point represents the average of nine datum points collected from three experiments performed in triplicate ($n = 9$). The *r* value presents only the seven linear datum points (B). A representative amplification plot showing the reproducibility of a triplicate experiment. Only the seven linear datum points are shown. ΔRn, fluorescence intensity; cycle, PCR cycle numbers.

concentration curve represents the average of nine C_T values (Fig. 1A). Even though DNA was detected throughout the entire dilution range, the linear range was approximately 6 logs (1×10^1 to 1.0×10^7 copies; Fig. 1A). An initial copy number of 10 copies yielded a C_T value of 37.9 ± 1.2 ($n = 9$), whereas an initial copy number of 1.0×10^7 copies yielded a C_T value of 18.5 ± 0.5 ($n = 9$; Fig. 1A.) As expected, every 10-fold decrease in the copy number yielded an increase in the C_T value of approximately 3, suggesting a linear relationship between the calculated and the initial DNA copy numbers (Perkin-Elmer, Foster City, Calif.). On the basis of the results of three experiments, the inter- and intra-assay variations were

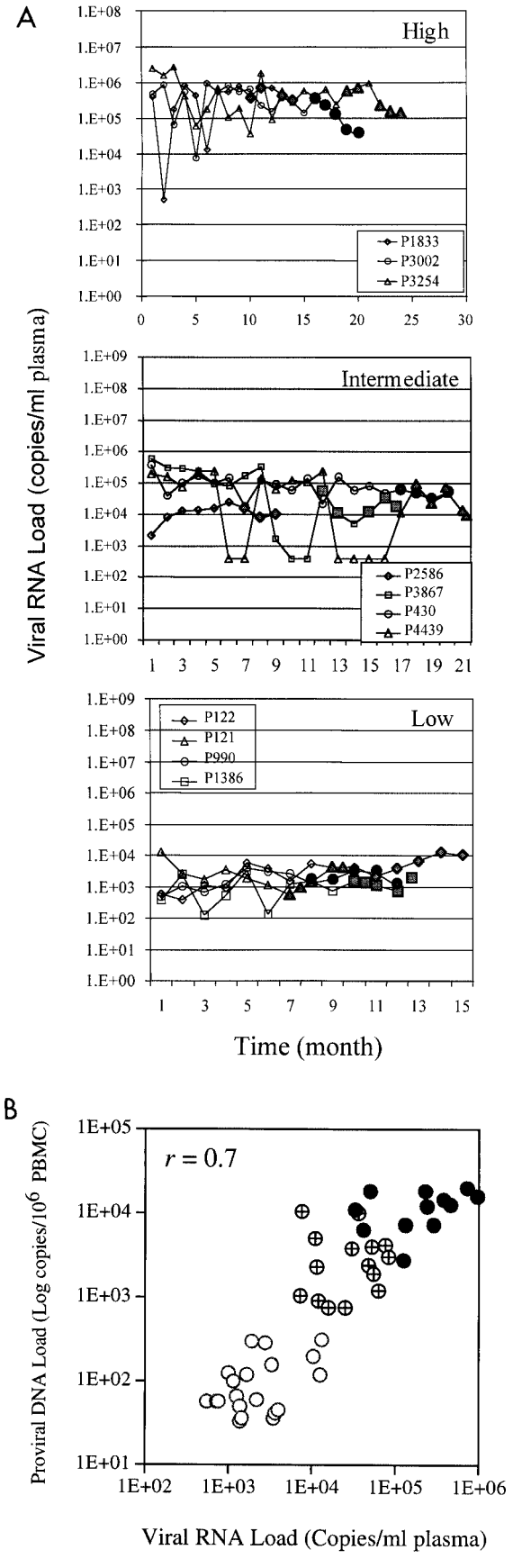

FIG. 2. Correlation of HIV-1 proviral DNA with viral RNA at stable stages. (A) Longitudinal measurement of viral RNA levels of HIV-infected children with viral RNA levels that are high (>5 $\log_{10}$ copies), intermediate (4 to 5) $\log_{10}$ copies), and low (2.6 to 4 $\log_{10}$ copies. Datum points with closed symbols represent results for samples for which the corresponding viral DNA level was measured by the TaqMan assay. (B) Positive correlation of the HIV-1 RNA level with the proviral DNA level when viral RNA levels were stabilized for at least 6 months.

TABLE 1. Assay validation by use of a blindly coded HIV-1 DNA quantification panel

Expected copy no.	Median estimates of actual copy no. based on[a]:	
	Cell counts (per 10^6 PBMCs)	DNA concn (per μg of DNA)
0	0	0
100	137	127
320	604	540
1,000	2,029	1,933

[a] Three samples with each expected copy number were tested.

within 2.5-fold over the entire dynamic range (for a representative plot, see Fig. 1B).

A PCR copy number panel, which was provided by the NIH AIDS Research and Reagent Reference Program, was used to test the detection limits of this assay. For 10 copies, HIV-1 *gag* DNA was detected 92% of the time (45 of 49 tests). For 5, 2.5, and 1.25 input copies of the *gag* gene, the detection frequencies were 83% (15 of 18 tests), 44% (8 of 18 tests), and 33% (6 of 18 tests), respectively, and the measured copy numbers were 5.2 ± 4.1, 1.9 ± 3.4, and 1.7 ± 3.5 copies, respectively. No *gag* DNA was detected in any *gag*-negative controls ($n = 43$), suggesting that this assay has a high degree of specificity.

A blindly coded HIV DNA quantification panel, which was provided by the Virological Quality Assurance program of the National AIDS Clinic Trials Group, was used to validate this assay. This panel consisted of 12 coded peripheral blood mononuclear cell (PBMC) samples that contained specified copy numbers including 3 HIV-negative specimens and 3 specimens each with 100, 320, and 1,000 copies per 10^6 PBMCs. Each sample was tested in triplicate three different times. The HIV-1 proviral DNA level was calculated as either the copy number per 10^6 cells or the number of micrograms of DNA. There was a close agreement (less than twofold) between the expected copy numbers and the actual copy numbers estimated on the basis of both the cell counts and the DNA concentration (Table 1). Even though estimates based on the cell counts were slightly higher than those based on the DNA concentration, there was no significant difference in the precision or the accuracy between these two estimates ($P = 0.44$) (Table 2). Therefore, both estimates can be used reliably to quantify HIV-1 proviral DNA.

By using this method, HIV-1 proviral DNA levels were compared in parallel with viral RNA levels in a total of 80 specimens from 18 HIV-infected children who were stratified into four groups on the basis of their viral RNA levels (Fig. 2A), i.e., children with high (>5 $\log_{10}$ copies), intermediate (4 to 5 $\log_{10}$ copies), low (2.6 to 4 $\log_{10}$ copies), and nondetectable

TABLE 2. Difference between expected and actual copy numbers (accuracy)[a]

Method	Difference between expected and actual copy no.			
	Minimum	Median	Maximum	SD
Cell counts	0.06	0.29	0.61	0.20
DNA concn	0.08	0.26	0.72	0.24

[a] The difference between the two methods (precision) was 0.02 ± 0.08 (mean ± standard deviation; $P = 0.44$; $n = 9$).

(<2.6 $\log_{10}$ copies) viral RNA levels. The inclusion criteria for each subject were that each had at least four consecutive samples with RNA levels within the defined range over a 6-month period. In addition, fluctuation of the viral RNA load during the 6-month period must have been less than 1 log. As shown in Fig. 2B, HIV-1 proviral DNA levels were positively correlated with the viral RNA load in the groups with high, intermediate, and low viral RNA levels ($r = 0.7$). Interestingly, of 18 samples that had nondetectable viral RNA for more than 6 months, HIV-1 proviral DNA was detected in all four patients, with only 3 of 18 samples (17%) having nondetectable viral DNA. The 15 DNA-positive samples had HIV-1 proviral DNA levels that varied over a range of approximately 2 logs, from 60 to 1,908 copies per 10^6 PBMCs. This observation suggests that proviral DNA could potentially be used as an additional surrogate marker to monitor the efficacy of antiretroviral therapy.

In summary, we developed and validated a real-time PCR (TaqMan) assay for quantification of HIV-1 proviral DNA. This assay is highly specific, with an analytic sensitivity of 10 viral DNA copies and a linear dynamic range of more than 6 logs (Fig. 1A). This assay is also reproducible, with intra- and interassay variabilities of <2.5-fold over the entire linear range (Fig. 1B). Since this method has a much broader linear dynamic range of detection than the conventional PCR assays, it should provide a useful means for quantification of the HIV-1 proviral DNA load in HIV-infected patients receiving antiviral therapy.

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