JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1996, p. 3196–3202 Vol. 34, No. 12
0095-1137/96/$04.00+0

An Enhanced-Sensitivity Branched-DNA Assay for Quantification of Human Immunodeficiency Virus Type 1 RNA in Plasma

DAVID KERN,[1] MARK COLLINS,[1] TIM FULTZ,[1] JILL DETMER,[1] SARAH HAMREN,[1] JOANNA J. PETERKIN,[2] PAT SHERIDAN,[1] MICKEY URDEA,[1] ROSEANN WHITE,[1] TORANGE YEGHIAZARIAN,[1] AND JOHN TODD[1]*

Chiron Corporation, Emeryville, California 94608-2916,[1] and Agouron Pharmaceuticals, Inc., La Jolla, California 92037-1020[2]

Received 10 June 1996/Returned for modification 9 August 1996/Accepted 4 September 1996

The quantification of human immunodeficiency virus type 1 (HIV-1) RNA has facilitated clinical research and expedited the development of antiretroviral drugs. The branched-DNA (bDNA) assay provides a reliable method for the quantification of HIV-1 RNA in human plasma and is considered one of the most reproducible assays ready for use in clinical trials. A series of oligonucleotide probe design and solution changes have been developed to enhance the sensitivity of the bDNA assay while maintaining its performance characteristics. Among the changes incorporated into the enhanced-sensitivity bDNA (ES bDNA) assay to reduce the background level and enhance the signal are the use of shorter overhang sequences of target probes for capture, the cruciform design of target probes for amplification, and the addition of preamplifier molecules. The ES bDNA assay is at least 20-fold more sensitive than the first-generation bDNA assay, yet it maintains a high level of accuracy, linearity, and reproducibility. Further, quantification values obtained with the ES bDNA assay and the first-generation bDNA assay are highly correlated, thus allowing for meaningful comparisons of HIV-1 RNA levels in specimens tested with either assay. The ES bDNA assay may be useful in determining the prognostic value of HIV-1 RNA levels of below 10,000 copies per ml and in assessing the clinical benefit of antiretroviral therapy-induced decreases in plasma HIV-1 RNA sustained at levels of below 10,000 copies per ml.

The quantification of human immunodeficiency virus type 1 (HIV-1) RNA in human plasma has facilitated clinical research and expedited the development of antiretroviral drugs. As a direct measure of viral burden, quantification of plasma HIV-1 RNA meets several requirements for HIV-1 infection markers. Levels of plasma HIV-1 RNA are associated with HIV-1 disease stage and progression, have low biological variability, and are strongly correlated with other known prognostic markers (5, 7, 11, 14, 18, 23). Changes in plasma HIV-1 RNA levels gauge the activity of antiretroviral agents. Plasma HIV-1 RNA levels decrease in response to antiretroviral therapy (15, 26) and increase upon selection and proliferation of resistant virus or removal of drug therapy (1, 3, 8).

Recent advances in clinical research and the development of more potent antiretroviral agents have generated new questions concerning the clinical relevance of low plasma HIV-1 RNA levels. Multivariate analysis has shown that the presence of high levels of plasma HIV-1 RNA (greater than 100,000 copies per ml) is the strongest predictor of rapid disease progression (20), yet the relationship between plasma HIV-1 RNA levels of below 10,000 copies per ml and disease progression is less clear. Studies monitoring the responses of subjects with a wide range of $CD4^+$ counts to investigational drugs have shown reductions in plasma HIV-1 RNA levels over a range of 2 to 3 $\log_{10}$ units (2, 6, 12, 13, 19). However, it is not known whether these drugs will have a comparable impact on plasma HIV-1 RNA levels in asymptomatic subjects, in whom pretreatment plasma HIV-1 RNA levels tend to be lower. Sensitive assays that measure small amounts of HIV-1 RNA are needed in the clinical research arena to address these issues.

Branched-DNA (bDNA) technology provides a novel approach for the quantification of plasma HIV-1 RNA. A significant departure from target amplification methods (1, 15, 21, 23), the bDNA assay directly measures HIV-1 RNA by boosting the reporter signal and thus avoids the errors inherent in the extraction and replication of target sequences. The bDNA assay is based on the hybridization of HIV-1 RNA to oligonucleotide probes complementary to the most conserved regions of the HIV-1 *pol* gene and yields highly specific, reproducible quantification of HIV-1 RNA that is not affected by the sequence variability of HIV-1 subtypes (22, 27, 28). Recently evaluated by the Quantitative Virology Working Group of the National Institute of Allergy and Infectious Diseases AIDS Clinical Trials Group (ACTG), the bDNA assay was considered one of the most reproducible HIV-1 RNA quantification assays ready for use in clinical trials (17).

Since the development of the first-generation bDNA assay (Quantiplex HIV RNA 1.0; Chiron Corporation, Emeryville, Calif.), we have continued to investigate improvements to bDNA technology. Our goal was to enhance the sensitivity of the bDNA assay while maintaining its performance characteristics. In this report, we describe a series of oligonucleotide probe design changes and concomitant improvements in the formulation of diluents and wash solutions that have been incorporated during the development of an enhanced-sensitivity bDNA (ES bDNA) assay. We analyze the sensitivity, linearity, and reproducibility of the ES bDNA assay and evaluate the effect of HIV-1 subtype diversity on HIV-1 RNA quantification. We also demonstrate the potential utility of the ES bDNA assay in the clinical research arena by monitoring changes in the virologic status of HIV-1-seropositive subjects undergoing therapy.

MATERIALS AND METHODS

Clinical specimens. Blood was collected by phlebotomy from HIV-1-seropositive and HIV-1-seronegative individuals into tubes containing EDTA and was stored at room temperature for up to 4 h. Plasma was separated by centrifugation

* Corresponding author. Mailing address: Chiron Corporation, 4560 Horton St., Emeryville, CA 94608-2916. Phone: (510) 601-3058. Fax: (510) 601-3307. Electronic mail address: john_todd@cc.chiron.com.

of whole blood at 800 × *g* for 10 min and stored at −70°C or lower. All specimens were coded and tested for HIV-1 RNA in a blinded fashion. Specimens obtained during an escalating-dose study of VIRACEPT kindly were provided by Agouron Pharmaceuticals, Inc., and were shipped frozen on dry ice to Chiron Corporation for testing. Informed consent was obtained from all specimen donors.

HIV-1 RNA transcripts. RNA transcripts containing a 2,829-bp fragment of the HIV-1 *pol* gene (nucleotides 2352 to 5181) were prepared by reverse transcription followed by PCR (RT-PCR) from cloned sequences representing HIV-1 subtypes A to F (28), using the MEGAscript in vitro transcription kit (Ambion, Austin, Tex.) for large-scale synthesis of RNA. In vitro-transcribed RNA was purified as described previously (4) and measured by three independent analytical methods, including optical density at 260 nm (10), phosphate determination (32), and hyperchromicity (31). The preparations of HIV-1 RNA transcripts used in this analysis were >97% pure by DE81 chromatography. Serial dilutions of transcripts in proteinase K-treated plasma were prepared for quantification of HIV-1 RNA in the ES bDNA assay.

Preparation of panels of potentially interfering substances. Panels of microorganisms were prepared by spiking HIV-1-seronegative plasma with 10^4 CFU of bacterial or yeast specimens per ml; the specimens included *Bacillus subtilis*, *Bacteroides fragilis*, *Citrobacter freundii*, *Enterobacter cloacae*, *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Peptostreptococcus magnus*, *Peptostreptococcus anaerobius*, *Propionibacterium acnes*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus* group B sp., *Yersinia enterocolitica*, and *Candida albicans*. Panels of viruses included hepatitis B virus DNA-positive serum (2.7×10^9 hepatitis B virus DNA equivalents per ml), hepatitis C virus RNA-positive serum (4×10^6 hepatitis C virus RNA equivalents per ml), and HIV-1-seronegative plasma seeded with cytomegalovirus-infected MRC5 cells (10^4 infected cells per ml).

Three pools of drugs commonly used in the management of HIV-1-infected patients were added to HIV-1-seronegative and HIV-1-seropositive plasma specimens. The drugs and their concentrations in plasma for pool 1 were 10 μg of zidovudine per ml, 10 μg of acyclovir per ml, 40 μg of fluconazole per ml, and 340 μg of trimethoprim-sulfamethoxazole per ml. The drugs and their concentrations in plasma for pool 2 were 2 μg of zalcitidine per ml, 30 μg of ganciclovir per ml, 350 μg of dapsone per ml, and 2.5 μg of rifabutin per ml. The drugs and their concentrations in plasma for pool 3 were 15 μg of vidanosine per ml, 20 μg of foscarnet per ml, 350 μg of ethambutol per ml, and 15 μg of clarithromycin per ml. All panels of specimens were frozen immediately after preparation and stored at −70°C until tested.

ES bDNA assay procedure. Plasma specimens, as well as positive and negative controls, were prepared in duplicate for the ES bDNA assay. Aliquots (1.0 ml) were measured into 1.5-ml conical tubes with O-ring screw cap seals (no. 772-692-005; Sarstedt, Newton, N.C.) and, after the addition of 50 μl of a 0.1% suspension of red polystyrene 0.5-μm-diameter beads (Bangs Laboratories, Carmel, Ind.) to aid in the visualization of virus pellets, were centrifuged at 23,500 × *g* for 1 h in a refrigerated microcentrifuge (centrifuge no. 17RS with rotor no. 3753; Heraeus, South Plainfield, N.J.). Supernatants were aspirated carefully, and the virus pellets either were processed immediately for the ES bDNA assay or were frozen at −70°C or colder. HIV-1 RNA was liberated from the virus pellets by addition of 220 μl of specimen diluent (100 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.5], 400 mM LiCl, 8 mM EDTA, 1% lithium lauryl sulfate, 12 μg of sonicated salmon sperm DNA per ml, 0.04% sodium azide, 0.04% Proclin 300 [Supelco, Bellefonte, Pa.], 2.2 mg of proteinase K per ml, 0.37 fmol of target probe set 1 per μl, 0.62 fmol of target probe set 2 per μl), followed by vortexing and incubation at 53°C for 20 min. Processed virus pellets were vortexed a second time and then held at room temperature for 5 to 15 min.

Capture of the HIV-1 RNA on the microwell surface was accomplished by adding 200-μl aliquots of each processed virus pellet to the appropriate oligonucleotide-modified microwell (24). For the standard curve which was run on every assay plate, 50-μl aliquots of standards A to H plus 150 μl of standards diluent (133 mM HEPES [pH 7.5], 532 mM LiCl, 10.6 mM EDTA, 1.3% lithium lauryl sulfate, 16 μg of sonicated salmon sperm DNA per ml, 0.04% sodium azide, 0.04% Proclin 300, 0.49 fmol of target probe set 1 per μl, 0.81 fmol of target probe set 2 per μl) were added to the appropriate wells on the same microplate. The microplate then was sealed with high-density polyethylene sheets under silicon pads and incubated overnight (12 to 16 h) at 53°C in a microwell plate heater (Chiron Corporation). The microwells were allowed to cool at room temperature for 10 min and then washed twice with wash A (0.1× SSC [1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate). After incubation at 53°C for 30 min with a 50-μl volume of preamplifier/amplifier diluent (prepared by incubating 50% horse serum, 1.3% sodium dodecyl sulfate, 6 mM Tris-HCl [pH 8.0], 5× SSC, and 0.5 mg of proteinase K per ml for 2 h at 65°C and then adding 6 mM phenylmethylsulfonyl fluoride, 0.05% sodium azide, and 0.05% Proclin 300) containing 0.70 fmol of preamplifier (described below) per μl, the microwells were cooled and washed as described above and then incubated at 53°C for 30 min with 50 μl of preamplifier/amplifier diluent containing 1.0 fmol of bDNA amplifier (29) per μl. After cooling and washing as described above, the microwells were incubated at 53°C for 15 min with a 50-μl volume of ES label diluent (preamplifier/amplifier diluent plus 0.85% Brij 35, 0.85 mM $ZnCl_2$, and 17 mM $MgCl_2$) containing 0.40 fmol of label probe (30) per μl. The microwells were cooled for 10 min and then washed twice with wash A and twice with wash D (0.1 M Tris-HCl [pH 8.0], 2.5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.1% Brij 35). A 50-μl volume of dioxetane substrate (Lumi-Phos Plus; Lumigen, Detroit, Mich.) was added to each microwell, and after incubation at 37°C for 30 min, the luminescent output was measured by photon counting in a plate reading luminometer (Chiron Corporation).

The amount of HIV-1 RNA in each specimen was quantified by using a standard curve. The assay standard consisted of a single-stranded DNA molecule, encoding the *gag* and *pol* genes of HIV-1_{SF2} (25), inserted into a phagemid vector which was assigned a value by comparison with quality level 1 HIV-1 RNA reference material as described previously (4). The single-stranded DNA standard was serially diluted in recalcified plasma from HIV-1-seronegative subjects (Base Matrix; Boston Biomedica, Inc., Boston, Mass.) to generate an eight-point standard curve, as follows (values are HIV-1 RNA copies per milliliter): standard A, 1.6×10^6; standard B, 5.3×10^5; standard C, 1.8×10^5; standard D, 5.9×10^4; standard E, 2.0×10^4; standard F, 6.6×10^3; standard G, 2.2×10^3; and standard H, 7.0×10^2. A calibration curve was generated from a least-squares quadratic polynomial fit in which the dependent variable was the $\log_{10}$ of the signal minus noise and the independent variable was the $\log_{10}$ of the HIV-1 RNA quantification value assignment for each standard. Signal-minus-noise values for both the test samples and standards were calculated by subtracting the geometric mean relative luminescence of two wells containing only Base Matrix from the relative luminescence of each well containing either a sample or a standard. HIV-1 RNA quantification values for each test sample were determined by calculating the mean $\log_{10}$ of the signal-minus-noise value, solving the quadratic equation for the $\log_{10}$ of the HIV-1 RNA quantification value, and then inverting back to the arithmetic scale. HIV-1 RNA quantification values were expressed in copies, where one copy was defined as the amount of HIV-1 RNA in a sample that generates a level of light emission equivalent to that generated by one copy of quality level 1 HIV-1 RNA reference material (4).

Synthesis of preamplifier molecules. Two preamplifier molecules, preamp I and preamp II, which differ only in the sequences complementary to target probe set 2, were synthesized. Preamp I consists of 237 bases and was constructed by enzymatic ligation of three oligomers (86, 79, and 73 bases) by using synthetic linkers. Preamp II consists of 239 bases and was constructed in a manner identical to that used for preamp I synthesis, with the exception that an 88-base oligomer was substituted for the 86-base oligomer. Hybridization and ligation were carried out overnight with T4 ligase (Pharmacia, Piscataway, N.J.) under standard conditions, and full-length products were purified by 10% denaturing polyacrylamide gel electrophoresis. Each preamplifier contains a site for hybridization with sequential overhang sequences of target probe set 2 and eight sites for hybridization with bDNA amplifier (29). The preamplifier used in the ES bDNA assay consists of an equimolar mixture of preamp I and preamp II.

Nucleic acid sequences. The nucleic acid sequences of the target probes in sets 1 and 2 which hybridize to the *pol* region of HIV-1 RNA are identical to those of the first-generation bDNA assay for HIV-1 RNA quantification described previously (22). The overhang sequences of the target probe sets have been shortened such that the target probes in set 1 contain a common 16-base overhang sequence (5′-CTCTTGGAAAGAAAGT-3′) and the target probes in set 2 contain 15-base overhang sequences that hybridize to the preamplifier molecules (see "Principles of the ES bDNA assay" in Results). The sequences of the preamplifier molecules are as follows: preamp I, 5′-AGGCATAGGACCCGTG TCTTTTTTAGGCATAGGACCCGTGTCTTTTTTAGGCATAGGACCCGT GTCCGTGGATGTTTGAGGCATAGGACCCGTGTCTTTTTTAGGCATA GGACCCGTGTCTTTTTTAGGCATAGGACCCGTGTCGCGTAGTGACT GAGGCATAGGACCCGTGTCTTTTTTAGGCATAGGACCCGTGTCTTTT TTCATATTCAAACTTTCGAGCCAGAAACTCAGT-3′; preamp II, 5′-AGG CATAGGACCCGTGTCTTTTTTAGGCATAGGACCCGTGTCTTTTTTAG GCATAGGACCCGTGTCCGTGGATGTTTGAGGCATAGGACCCGTGTC TTTTTTAGGCATAGGACCCGTGTCTTTTTTAGGCATAGGACCCGTGT CGCGTAGTGACTGAGGCATAGGACCCGTGTCTTTTTTAGGCATAGG ACCCGTGTCTTTTTTAGGTAGGTAGGTAGGTGACTGACTGACTGAC T-3′.

Statistical analysis. The percent coefficient of variance (CV) was based on a pooled variance estimate across operators. The variance estimates were calculated by using analysis-of-variance techniques as described previously (28). The 95% prediction limits were calculated by using a linear model (9).

RESULTS

Principles of the ES bDNA assay. An ES bDNA assay was developed by modifying the Quantiplex HIV RNA 1.0 assay originally described by Pachl et al. (22) to reduce the background level and increase the number of bDNA amplifier molecules used to generate the signal. This was accomplished through the addition of preamplifier molecules which require specific alignment of oligonucleotide sequences and contain eight bDNA amplifier hybridization sites. Like the Quantiplex HIV RNA 1.0 assay, the ES bDNA assay uses a solution-phase

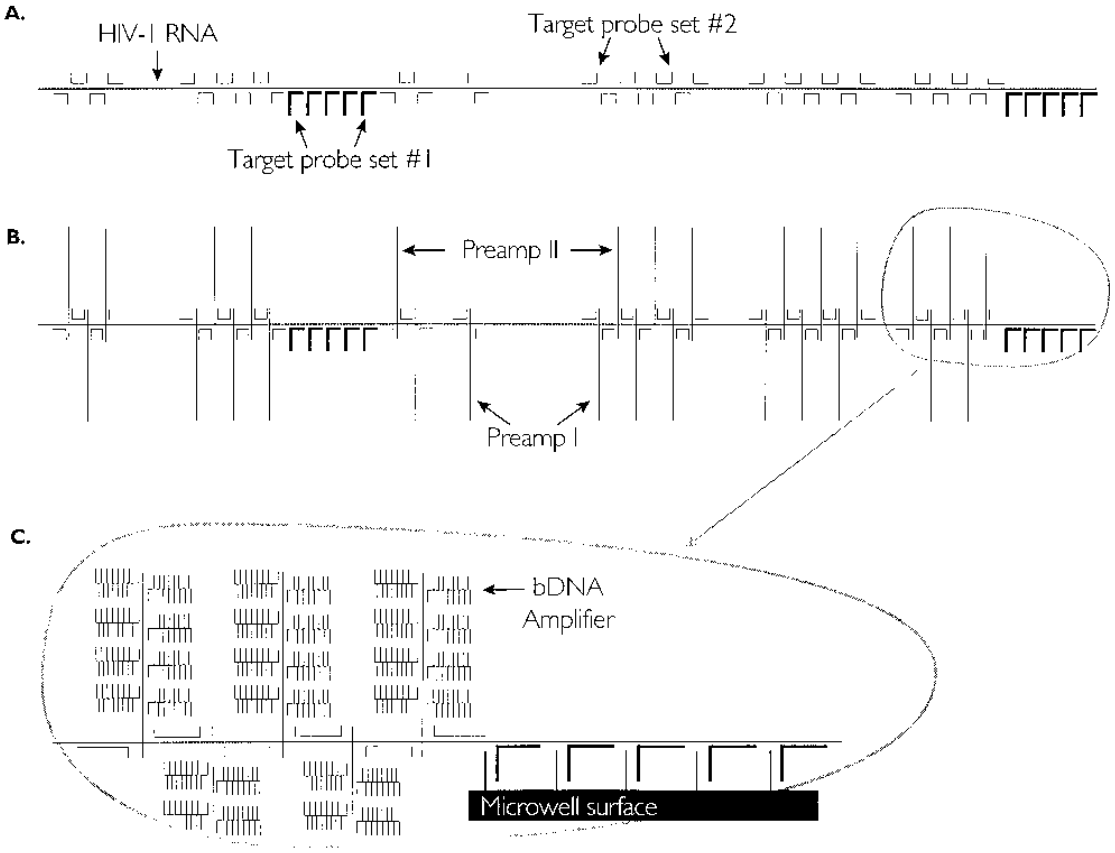

FIG. 1. Schematic representation of the ES bDNA assay for quantification of HIV-1 RNA. (A) Target probes hybridize to unique 33-base sequences at different positions along the conserved region of the HIV-1 *pol* gene. Target probe set 1 mediates capture of the HIV-1 RNA to the microwell surface, whereas target probe set 2 mediates preamplifier binding. (B) Neighboring target probes are bridged by preamplifier molecules (preamp I and preamp II). (C) Enhancement of the signal is accomplished by the binding of up to eight bDNA amplifier molecules to each preamplifier and of 45 alkaline phosphatase-conjugated label probes to each bDNA amplifier molecule.

sandwich assay format in which HIV-1 RNA is hybridized in solution with oligonucleotide target probes containing a unique 33-base sequence that hybridizes to a conserved region of the HIV-1 *pol* gene. A total of 45 target probes were designed, including 10 to mediate binding of the HIV-1 RNA to capture probes on the microwell surface (target probe set 1) and 35 to mediate binding of the HIV-1 RNA to preamplifier molecules (target probe set 2). Figure 1A illustrates the placement of the target probes by position, where each position represents a unique 33-base sequence. The first position starts at nucleotide 244 and the last position ends at nucleotide 2833 in the *pol* gene of model HIV strain SF2 described by Gerald Myers (Los Alamos sequence database). Each of target probes in set 1 contains a common 16-base overhang sequence that hybridizes to the capture probes on the microwell surface. As shown in Fig. 1B, the design of target probe set 2 is such that two target probes must be bound to adjacent regions of the HIV-1 RNA for efficient hybridization to the preamplifier molecule to occur. The longer sequence established by the binding of two sequential overhang sequences of target set 2 stabilizes the hybridization of the preamplifier molecule into a cruciform hybrid resembling a Holiday junction noted during DNA recombination. By design, hybridization of the preamplifier to a shorter overhang sequence alone is thermodynamically unstable. Two preamplifier molecules were designed to bridge neighboring target probes. They contain the same repeat sequence and differ only in the sequences that hybridize to the sequential overhang regions of target probe set 2 (preamp I, 5′-CATATTCAAACTTTCGAGCCAGAAACTCAGT-3′; preamp II, 5′-AGGTAGGTAGGTAGGTGACTGACTGAC TGACT-3′). The 35 probes in target probe set 2 can bind up to 14 preamp I molecules and 14 preamp II molecules. As shown in the magnified view in Fig. 1C, each preamplifier molecule can bind up to eight bDNA amplifier molecules by hybridization to complementary 18-base sequences, and each bDNA amplifier molecule contains 15 branches, each of which can bind three alkaline phosphatase-conjugated label probes. Thus, at the end of the hybridization steps, each captured HIV-1 RNA molecule may be decorated with as many as 10,080 separate alkaline phosphatase-conjugated label probes.

Analytical quantification limit and linearity. The analytical quantification limit of the ES bDNA assay was determined by testing twofold serial dilutions of assay standard A in recalcified HIV-1 seronegative plasma (Base Matrix). Four replicates of each dilution as well as a negative control, Base Matrix without added standard A, were tested within one assay run. The relative luminescence obtained for the negative control was subtracted from the relative luminescence obtained for each dilution (signal minus noise), and the resulting relationship is shown in Fig. 2A. A one-tailed Dunett's *t* test indicated that the lowest concentration that could be distinguished as significantly different from the negative control ($P < 0.05$) was 390 copies per ml.

The linearity of the ES bDNA assay was evaluated by testing

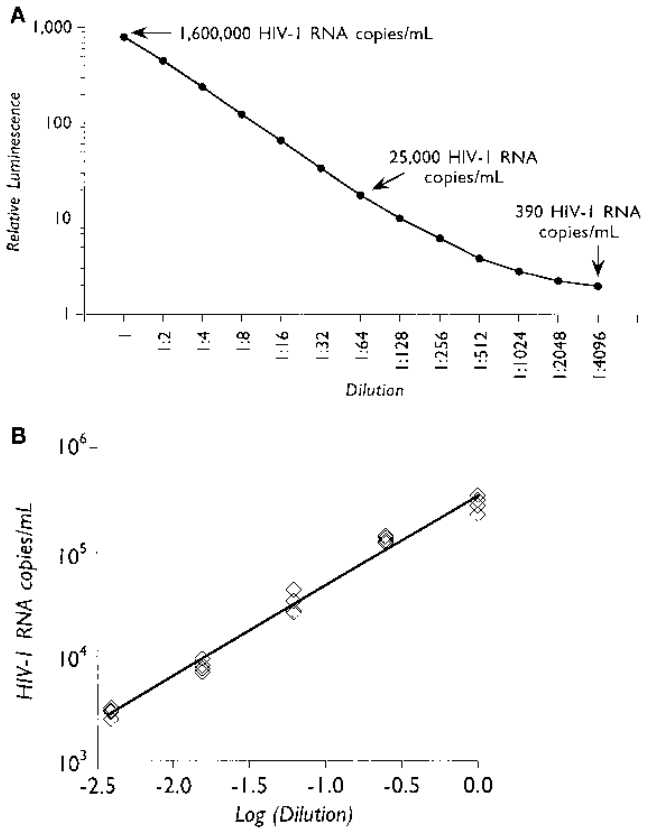

FIG. 2. (A) Twofold dilution series evaluating the analytical quantification limit of the ES bDNA assay. (B) Fourfold dilution series evaluating the linearity of the ES bDNA assay.

fourfold serial dilutions of an HIV-1-seropositive plasma specimen prepared in HIV-1-seronegative plasma. Four replicates of each dilution were tested during the same assay run, and the results are shown in Fig. 2B. The relationship between HIV-1 RNA quantification values and dilutions can be defined by the equation $y = 5.54 + 0.87x$. An r^2 value of 0.986 was calculated by linear regression analysis, indicating that ~99% of the observed variation can be explained by the linear relationship between dilutions of HIV-1 RNA-positive specimens and quantification values. These results also demonstrated that the ES bDNA assay yielded accurate quantification values for all dilutions tested, which covered most of the dynamic range of the standard curve.

Reproducibility. The reproducibility of the ES bDNA assay was established by testing replicates of specimen panels in 30 separate assay runs by five different operators (Table 1). The specimen panels included five HIV-1-seropositive specimens with HIV-1 RNA levels that spanned the range of the standard curve. The CV ranged from 17 to 39% for overall assay precision. These values reflect assay reproducibility encountered in real-time testing when specimens are tested on different days by different operators. As expected, lower CV values, ranging from 12 to 25%, were observed for within-assay precision. These values reflect the assay reproducibility encountered in batch testing, such as that commonly used in longitudinal clinical trials of antiviral agents. In this analysis, the variability was largest near the quantification limit, which is consistent with the criteria used to establish such a limit.

Reproducibility also was evaluated independently by using

TABLE 1. Reproducibility of the ES bDNA assay

Specimen no.	HIV-1 RNA copies/ml (geometric mean)	Assay precision (% CV)		
		Within run[a]	Within day[b]	Overall[c]
1	1,400	25	37	39
2	5,500	19	20	23
3	25,000	13	16	17
4	72,000	14	18	18
5	190,000	12	17	19

[a] Two determinations per plate, one plate, one operator, one day.
[b] Two determinations per plate, two plates, one operator, one day.
[c] Two determinations per plate, six plates, five operators, three days.

the ES bDNA assay to test panels of HIV-1 culture isolates diluted into HIV-1-seronegative serum provided by the ACTG Viral Quality Assurance Program (proficiency panel 03) (Table 2, panel A) and a panel of naturally occurring specimens provided by Bill Schleiff, Merck and Co., Inc., West Point, Pa. (Table 2, panel B). Samples were blinded at the time of testing, and the specimens were run in replicates of two to four. Results were reported to the ACTG Viral Quality Assurance Laboratory (Rush Presbyterian Hospital, Chicago, Ill.) and Merck, where they were decoded and analyzed. In these independent evaluations, the assay reproducibility was consistent with that reported in Table 1. Analysis of these data to calculate the 95% prediction limits demonstrated that the reproducibility of the ES bDNA assay is sufficient to discern two- and threefold changes in HIV-1 RNA levels as statistically significant for tests in batch and real-time modes, respectively.

Correlation with Quantiplex HIV RNA 1.0 assay. As shown in Fig. 3, the relationship between quantification values measured with the Quantiplex HIV RNA 1.0 assay and those measured with the ES bDNA assay was explored by testing multiple plasma specimens. The Pearson's correlation coefficient (r value) was calculated to be 0.96, indicating that the quantification values measured with the Quantiplex HIV RNA 1.0 and ES bDNA assays were highly correlated.

The correlation between the two assays also was evaluated by testing serial dilutions of HIV-1-seropositive plasma in HIV-1-seronegative plasma. Three replicates of each of four dilutions, covering a range of approximately 2 $\log_{10}$ units, were

TABLE 2. Independent evaluation of the reproducibility of the ES bDNA assay

Panel[a] and specimen no.	No. of replicates	HIV-1 RNA copies/ml (mean)	Precision[b] (% CV)
A			
1	3	1,400	6
2	3	5,900	6
3	3	28,200	11
4	3	135,800	11
5	3	632,800	12
B			
1	3	830	14
2	2	2,900	34
3	2	10,100	11
4	2	11,200	2
5	4	33,200	7

[a] Panel A was provided through the ACTG Virology Quality Assurance Program; panel B was provided by Merck and Company, Inc.
[b] All specimens were tested in the batch mode.

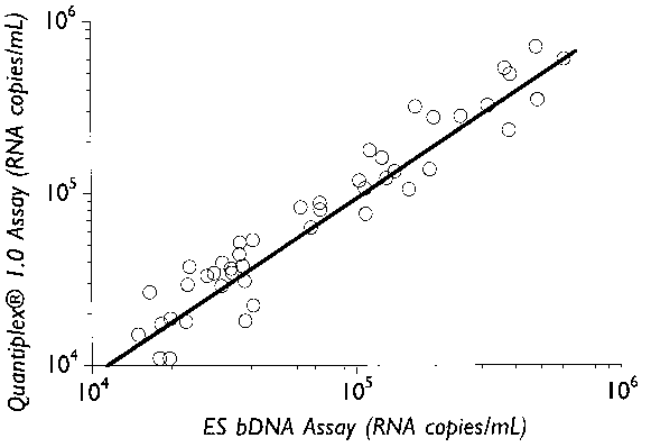

FIG. 3. Correlation between quantification values obtained with the Quantiplex HIV RNA 1.0 assay and those measured with the ES bDNA assay (r = 0.96).

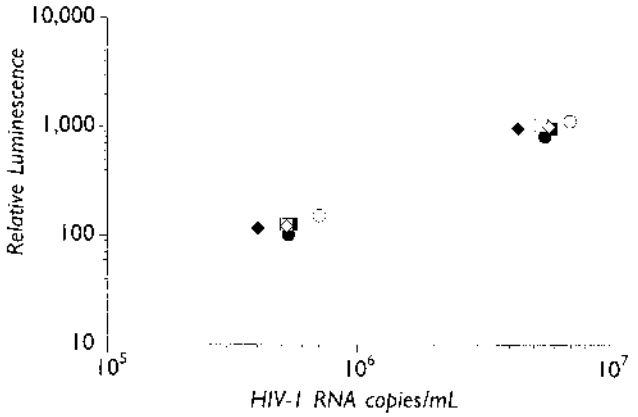

FIG. 4. Dilutions of RNA transcripts representing HIV-1 subtypes A (●), B (○), C (■), D (□), E (◆), and F (◇), measured with the ES bDNA assay.

tested with the Quantiplex HIV RNA 1.0 and ES bDNA assays (data not shown). In this experiment, an r value of 0.995 was calculated, further supporting a high degree of correlation between the quantification values measured with the Quantiplex HIV RNA 1.0 and ES bDNA assays.

Effect of potentially interfering substances. To test whether the ES bDNA assay was specific for RNA from HIV-1 and did not react with nucleic acids or components of other viruses or other microorganisms which might be found in blood, hepatitis B virus DNA-positive serum specimens, hepatitis C virus RNA-positive serum specimens, HIV-1-seronegative plasma specimens seeded with cytomegalovirus-infected MRC5 cells, and a multitude of bacterial and yeast cultures were tested. In all cases the quantification values produced were below the quantification limit of the ES bDNA assay. To further evaluate the effect of potentially interfering microorganisms, HIV-1-seropositive and HIV-1-seronegative plasma specimens seeded with cultures of bacteria commonly associated with HIV-1 disease were tested with the ES bDNA assay (Table 3). Quantification values below the limit of the ES bDNA assay were observed for all HIV-1-seronegative specimens tested. HIV-1-seropositive specimens with and without added bacteria showed similar HIV-1 RNA levels. A paired t-test analysis showed no significant difference in quantification values between samples ($P > 0.05$).

TABLE 3. Effect of common microorganisms and drugs on the performance of the ES bDNA assay

Microorganism or drug pool	HIV-1 RNA copies/ml (geometric mean)[a] in:			
	HIV-1-seronegative plasma		HIV-1-seropositive plasma	
	Alone	Seeded	Alone	Seeded
Microorganisms				
Cryptococcus neoformans	<500	<500	2,800	2,700
Mycobacterium avium	<500	<500	3,400	3,400
Streptococcus pneumoniae	<500	<500	2,800	2,800
Drug pools[b]				
1	<500	<500	3,100	3,200
2	<500	<500	4,100	4,000
3	<500	<500	3,400	3,500

[a] In each case, the difference between the values for seeded and unseeded samples was not significant ($P > 0.05$).
[b] The compositions of the drug pools are given in Materials and Methods.

The performance of the ES bDNA assay in the presence of therapeutic and prophylactic drugs commonly used in the management of HIV-1-infected patients was evaluated (Table 3). The drugs were combined into three pools and added to HIV-1-seronegative and HIV-1-seropositive plasma specimens at concentrations that exceed pharmokinetic peak levels in plasma by fivefold. All HIV-1-seronegative specimens produced quantification values below the limit of the ES bDNA assay, and comparable HIV-1 RNA levels were measured in HIV-1-seropositive specimens with and without added drugs. No significant difference in quantification values between samples was indicated by paired t-test analysis.

Effect of HIV-1 genotypic variation. The effect of HIV-1 genotypic variation on HIV-1 RNA quantification by the ES bDNA assay was assessed by testing serial dilutions of quality level 2 RNA transcripts (4) representing *pol* gene sequences from HIV-1 subtypes A to F. As shown in Fig. 4, HIV-1 RNAs from all six subtypes were quantified equally by the ES bDNA assay over a range of at least 2 $\log_{10}$ units. A 1.4-fold difference in quantification, between HIV-1 subtypes B and E, was the maximum variance observed. This level of accuracy has been difficult to attain with other methodologies (27).

Quantification of HIV-1 RNA in patients undergoing therapy. The ES bDNA assay was used to monitor plasma HIV-1 RNA levels in six patients treated with VIRACEPT (AG1343; nelfinavir mesylate), a novel HIV protease inhibitor (Fig. 5). A rapid decrease in HIV-1 RNA levels was observed by day 4 after the initiation of therapy, with the peak response occurring at days 7 and 14. The response was substantial: a decrease in plasma HIV-1 RNA levels of 2 to 2.5 $\log_{10}$ units was noted in all patients. Also, plasma HIV-1 RNA levels were driven to below 500 copies per ml in all patients at some time during therapy, irrespective of baseline plasma HIV-1 RNA levels. Further, the response was sustained throughout the monitoring period: plasma HIV-1 RNA levels remained low in four patients monitored to 28 days and in two patients monitored to 60 days.

DISCUSSION

With the development of new antiretroviral agents used to treat patients with HIV-1 infection, new questions have emerged concerning the clinical relevance of low plasma

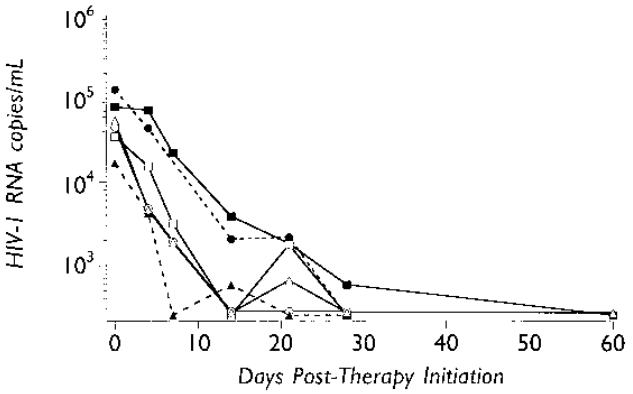

FIG. 5. Changes in plasma HIV-1 RNA levels in six subjects treated with VIRACEPT (AG1343), measured with the ES bDNA assay. Dosages ranged from 500 to 1,000 mg two or three times per day as follows: patient 1, 750 mg three times per day, (●); patient 2, 750 mg three times per day (○); patient 3, 750 mg two times per day (■); patient 4, 500 mg three times per day (□); patient 5, 1,000 mg three times per day (▲); and patient 6, 600 mg three times per day (△).

HIV-1 RNA levels. Reliable and sensitive assays to measure low plasma HIV-1 RNA levels are needed to address important clinical research issues. For example, assays yielding accurate quantification of HIV-1 RNA at below 10,000 copies per ml may be useful in clinical research to evaluate the relationship between low plasma HIV-1 RNA levels and disease progression. Studies examining the impact of investigational drugs on plasma HIV-1 RNA levels in asymptomatic patients also may benefit from the availability of more sensitive, quantitative assays.

We have developed a number of oligonucleotide probe design and solution changes for the bDNA assay to reduce the background level and to enhance the signal for quantification of HIV-1 RNA in plasma. Among the changes incorporated into the ES bDNA assay are the shorter overhang sequences of target probes for capture (target probe set 1), the cruciform design of target probes for amplification (target probe set 2), and the addition of preamplifier molecules. The shorter overhang sequences of target probe set 1 decrease the T_m for hybridization of target probe set 1 to HIV-1 RNA by ~12°C. By relying on the concatenation of nearby probes hybridized to the HIV-1 RNA to increase the T_m, nonspecific hybridization of the target probes is diminished and background noise is reduced. The assay background level is decreased further by the cruciform design of target probe set 2. The overhang sequences of target probe set 2 are 15 or 16 bases in length and individually cannot efficiently bind to the preamplifier. However, when the overhang sequences of two target probes are adjacent, the T_m increases, thereby stabilizing the hybridization of the preamplifier. In addition to the reduction of background noise, the signal generated by the specific binding of HIV-1 RNA is increased by including preamplifier molecules, each of which contains eight sites for hybridization with bDNA amplifier molecules.

In designing the ES bDNA assay, accuracy was of paramount importance. Models for the prediction of disease progression and likelihood of response to therapy are based on studies of populations of patients in which HIV-1 RNA levels are measured. Thus, to be clinically meaningful, it is imperative that HIV-1 RNA quantification values be accurate. The ES bDNA assay therefore includes standards run in duplicate on each plate that are assigned values in comparison with HIV-1 RNA reference standards aligned with the U.S. National Institute of Standards and Technology phosphate standard (4). Positive and negative controls also are run on each plate to verify preset quantification limits. In addition, specimens are run in duplicate so that outliers can be readily identified. Another important consideration in designing the ES bDNA assay was ease of use in a clinical setting. In the ES bDNA assay, the second centrifugation step of the Quantiplex HIV RNA 1.0 assay has been eliminated, thus simplifying the procedure and enabling a high throughput. With the ES bDNA assay, one person can run three plates yielding 126 determinations within 24 h.

Our results show that the ES bDNA assay is at least 20-fold more sensitive than the Quantiplex HIV RNA 1.0 assay yet maintains the performance characteristics of the Quantiplex HIV RNA 1.0 assay with regard to accuracy, linearity, and reproducibility. Further, our results demonstrate that the quantification values obtained with the ES bDNA assay and the Quantiplex HIV RNA 1.0 assay are highly correlated. The high degree of correlation between quantification values allows for meaningful comparisons of HIV-1 RNA levels in specimens measured with either bDNA assay. For example, it may be appropriate first to test all specimens with the Quantiplex HIV RNA 1.0 assay and then to retest only those specimens with HIV-1 RNA levels of below 10,000 copies per ml with the ES bDNA assay. The specimen volume requirement of the ES bDNA assay (1 ml) may be limiting for studies involving low-volume specimens. Ongoing experiments are aimed at lowering the specimen volume requirement of the ES bDNA assay. In preliminary experiments, we have tested 50-μl specimen volumes without centrifugation in the ES bDNA assay by adding plasma directly to the microplate wells and have obtained excellent HIV-1 RNA recovery and quantification compared with those for the 1-ml assay format. With the 50-μl format, however, comes a concomitant 20-fold decrease in assay sensitivity on a per-milliliter basis. Nevertheless, this 50-μl format for the ES bDNA assay may be especially useful for the low-volume specimens in retrospective studies as well as for studies using specimens from newborns (16).

The use of the ES bDNA assay to monitor plasma HIV-1 RNA levels in patients undergoing therapy in this and other studies (12, 19, 33) illustrates the potential utility of this assay in clinical research to evaluate the effectiveness of antiretroviral agents. With the lower clinical quantification limit, this assay may be useful in determining the prognostic value of HIV-1 RNA levels of below 10,000 copies per ml and in assessing the clinical benefit of antiretroviral therapy-induced decreases in plasma HIV-1 RNA sustained at levels of below 10,000 copies per ml.

ACKNOWLEDGMENTS

We thank Bill Schleiff at Merck and Co., Inc., and Brian Staes at the ACTG Viral Quality Assurance Program for reproducibility panels. We appreciate the efforts of Kristina Whitfield for graphic artwork, Linda Wuestehube for editorial assistance, and Judy Wilber and David Chernoff for critical review of the manuscript. Finally, we extend our thanks to the dedicated individuals of the Chiron Reference Testing Laboratory team for their excellent technical assistance.

REFERENCES

1. **Aoki-Sei, S., R. Yarchoan, S. Kageyama, D. T. Hoekzema, J. M. Pluda, K. M. Wyvill, S. Broder, and H. Mitsuya.** 1992. Plasma HIV-1 viremia in HIV-1 infected individuals assessed by polymerase chain reaction. AIDS Res. Hum. Retroviruses **8:**1263–1270.
2. **Biron, F., F. Lucht, D. Peyramond, A. Fresard, T. Vallet, F. Nugier, J. Grange, S. Mailey, F. Hamedi-Sangsaru, and J. Vila.** 1995. Anti-HIV activity of the combination of didanosine and hydroxyurea in HIV-1-infected individuals. J. Acquired Immune Defic. Syndr. Hum. Retrovirol. **10:**36–40.
3. **Cao, Y., D. D. Ho, J. Todd, R. Kokka, M. Urdea, J. D. Lifson, M. J. Piatak,**

S. Chen, B. H. Hahn, M. S. Saag, and G. M. Shaw. 1995. Clinical evaluation of branched DNA (bDNA) signal amplification for quantifying HIV-1 in human plasma. AIDS Res. Hum. Retroviruses **11:**353–361.

4. **Collins, M. L., C. Zayati, J. J. Detmer, B. Daly, J. A. Kolberg, T. A. Cha, B. D. Irvine, J. Tucker, and M. S. Urdea.** 1995. Preparation and characterization of RNA standards for use in quantitative branched DNA hybridization assays. Anal. Biochem. **226:**120–129.
5. **Connor, R. I., H. Mohri, Y. Cao, and D. D. Ho.** 1993. Increased viral burden and cytopathicity correlate temporally with $CD4^+$ T-lymphocyte decline and clinical progression in human immunodeficiency virus type 1-infected individuals. J. Virol. **67:**1772–1777.
6. **Danner, S. A., A. Carr, J. M. Leonard, L. M. Lehman, F. Gudiol, J. Gonzales, A. Raventos, R. Rubio, E. Bouza, V. Pintado, A. G. Aguado, J. G. De Lomas, R. Delgado, J. C. C. Borleffs, A. Hsu, J. M. Valdes, C. A. B. Boucher, and D. A. Cooper, for the European-Australian Collaborative Ritonavir Study Group.** 1995. A short-term study of the safety, pharmacokinetics, and efficacy of ritonavir, an inhibitor of HIV-1 protease. N. Engl. J. Med. **23:**1528–1533.
7. **Dewar, R. L., H. C. Highbarger, M. D. Sarmiento, J. A. Todd, M. B. Vasudevachari, R. J. Davey, J. A. Kovacs, N. P. Salzman, H. C. Lane, and M. S. Urdea.** 1994. Application of branched DNA signal amplification to monitor human immunodeficiency virus type 1 burden in human plasma. J. Infect. Dis. **170:**1172–1179.
8. **Eastman, P. S., M. Urdea, D. Besemer, M. Stempien, and J. Kolberg.** 1995. Comparison of selective polymerase chain reaction primers and differential probe hybridization of polymerase chain reaction products for determination of relative amounts of codon 215 mutant and wild-type HIV-1 populations. J. Acquired Immune Defic. Syndr. Hum. Retrovirol. **9:**264–273.
9. **Franklin, G.** 1976. Theory and application of the linear model, p. 606–648. Wadsworth Publishing Co., Belmont, Calif.
10. **Henderson, J. T., A. S. Benight, and S. Hanlon.** 1992. A semi-micromethod for the determination of the extinction coefficients of duplex and single-stranded DNA. Anal. Biochem. **201:**17–29.
11. **Henrard, D. R., J. F. Philips, L. R. Muenez, W. A. Blattner, D. Wiesner, E. Eyster, and J. J. Goedert.** 1995. Natural history of HIV-1 cell-free viremia. JAMA **274:**554–558.
12. **Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature (London) **373:**123–126.
13. **Ho, D. D., A. S. Perelson, and G. M. Shaw.** 1995. HIV results in the frame—reply. Nature (London) **375:**198.
14. **Hogervorst, E., S. Jurriaans, F. de Wolf, A. van Wijk, A. Wiersma, M. Valk, M. Roos, B. van Gemen, R. Coutinho, F. Miedema, and J. Goudsmit.** 1995. Predictors for non- and slow progression in human immunodeficiency virus (HIV) type 1 infection: low viral RNA copy numbers in serum and maintenance of high HIV-1 p24-specific but not V3-specific antibody levels. J. Infect. Dis. **171:**811–821.
15. **Holodniy, M., L. Mole, M. Winters, and T. C. Merigan.** 1994. Diurnal and short-term stability of HIV virus load as measured by gene amplification. J. Acquired Immune Defic. Syndr. **7:**363–368.
16. **Kabat, W., T. Yeghiazarian, J. Wilber, Y. Zhao, and R. Yogev.** 1995. Quantification of human immunodeficiency virus type 1 (HIV-1) RNA levels in pediatric population using a modified branched DNA signal amplification method, abstr. I161, p. 233. *In* Abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
17. **Lin, H. J., L. E. Myers, B. Yen-Lieberman, F. B. Hollinger, D. Henrard, C. J. Hooper, R. Kokka, S. Kwok, S. Rasheed, M. Vahey, M. A. Winters, L. J. McQuay, P. L. Nara, P. Reichelderfer, R. W. Coombs, and J. B. Jackson.** 1994. Multicenter evaluation of quantification methods for plasma human immunodeficiency virus type 1 RNA. J. Infect. Dis. **170:**553–562.
18. **Loveday, C., and A. Hill.** 1995. Prediction of progression to AIDS with serum HIV-1 RNA and CD4 count. Lancet **345:**790–791.
19. **Markowitz, M., M. Saag, W. G. Powderly, A. M. Hurley, A. Hsu, J. M. Valdes, D. Henry, F. Sattler, A. La Marca, J. M. Leonard, and D. D. Ho.** 1995. A preliminary study of ritonavir, an inhibitor of HIV-1 protease, to treat HIV-1 infection. N. Engl. J. Med. **23:**1534–1539.
20. **Mellors, J. W., L. A. Kingsley, C. R. J. Rinaldo, J. A. Todd, B. S. Hoo, R. P. Kokka, and P. Gupta.** 1995. Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion. Ann. Intern. Med. **122:**573–579.
21. **Mulder, J., N. McKinney, C. Christopherson, J. Sninsky, L. Greenfield, and S. Kwok.** 1994. Rapid and simple PCR assay for quantitation of human immunodeficiency virus type 1 RNA in plasma: application to acute retroviral infection. J. Clin. Microbiol. **32:**292–300.
22. **Pachl, C., J. A. Todd, D. G. Kern, P. J. Sheridan, S.-F. Fong, M. Stempien, B. Hoo, D. Besemer, T. Yeghiazarian, B. Irvine, J. Kolberg, R. Kokka, P. Neuwald, and M. S. Urdea.** 1995. Rapid and precise quantification of HIV-1 RNA in plasma using a branched DNA (bDNA) signal amplification assay. J. Acquired Immune Defic. Syndr. Hum. Retrovirol. **8:**446–454.
23. **Piatak, M. J., M. S. Saag, L. C. Yang, S. J. Clark, J. C. Kappes, K. C. Luk, B. H. Hahn, G. M. Shaw, and J. D. Lifson.** 1993. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. Science **259:**1749–1754.
24. **Running, J. A., and M. S. Urdea.** 1990. A procedure for productive coupling of synthetic oligonucleotides to polystyrene microtiter wells for hybridization capture. BioTechniques **8:**276–277.
25. **Sánchez-Pescador, R., M. D. Power, P. J. Barr, K. S. Steimer, M. M. Stempien, S. L. Brown-Shimer, W. W. Gee, A. Renard, A. Randolph, J. A. Levy, D. Dina, and P. A. Luciw.** 1985. Nucleotide sequence and expression of an AIDS-associated retrovirus (ARV-2). Science **227:**484–492.
26. **Semple, M., C. Loveday, I. Weller, and R. Tedder.** 1991. Direct measurement of viraemia in patients infected with HIV-1 and its relationship to disease progression and zidovudine therapy. J. Med. Virol. **35:**38–45.
27. **Todd, J., C. Pachl, R. White, T. Yeghiazarian, P. Johnson, B. Taylor, M. Holodniy, D. Kern, S. Hamren, D. Chernoff, and M. Urdea.** 1995. Performance characteristics for the quantitation of plasma HIV-1 RNA using branched DNA signal amplification technology. J. Acquired Immune Defic. Syndr. Hum. Retrovirol. **10:**S35–S44.
28. **Todd, J., T. Yeghiazarian, B. Hoo, J. Detmer, J. Kolberg, R. White, J. Wilber, and M. Urdea.** 1994. Quantitation of human immunodeficiency virus plasma RNA by branched DNA and reverse transcription coupled polymerase chain reaction assay methods: a critical evaluation of accuracy and reproducibility. Serodiagn. Immunother. Infect. Dis. **6:**1–7.
29. **Urdea, M. S., T. Horn, T. Fultz, M. Anderson, J. Running, S. Hamren, D. Ahle, and C.-A. Chang.** 1991. Branched DNA amplification multimers for the sensitive, direct detection of human hepatitis viruses. Nucleic Acids Res. Symp. Ser. **24:**197–200.
30. **Urdea, M. S., B. D. Warner, J. A. Running, M. Stempien, J. Clyne, and T. Horn.** 1988. A comparison of non-radioisotopic hybridization assay methods using fluorescent, chemiluminescent and enzyme labeled synthetic oligodeoxyribonucleotide probes. Nucleic Acids Res. **16:**4937–4956.
31. **Warshaw, M. M., and I. Tinoco, Jr.** 1966. Optical properties of sixteen dinucleoside phosphates. J. Mol. Biol. **20:**29–38.
32. **Webb, M. R.** 1992. A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems. Proc. Natl. Acad. Sci. USA **89:**4884–4887.
33. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, M. S. Saag, and G. M. Shaw.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature (London) **373:**117–126.