Exhibit B

Multicenter Evaluation of Quantification Methods for Plasma Human Immunodeficiency Virus Type 1 RNA

Hsiang Ju Lin, Lawrence E. Myers, Belinda Yen-Lieberman, F. Blaine Hollinger, Denis Henrard, Carol J. Hooper, Robert Kokka, Shirley Kwok, Suraiya Rasheed, Maryanne Vahey, Mark A. Winters, Lisa J. McQuay, Peter L. Nara, Patricia Reichelderfer, Robert W. Coombs, and J. Brooks Jackson

Division of Molecular Virology, Baylor College of Medicine, Houston, Texas; Research Triangle Institute, Research Triangle Park, North Carolina; Department of Pathology, Cleveland Clinic Foundation, University Hospitals of Cleveland, and Case Western Reserve University, Cleveland, Ohio; Abbott Laboratories, Abbott Park, Illinois; Retrovirus Laboratory, University of Washington, Seattle; Chiron Corp., Emeryville, Roche Molecular Systems, Alameda, Laboratory of Viral Oncology and AIDS Research, University of Southern California School of Medicine, Los Angeles, and Center for AIDS Research, Stanford University School of Medicine, Palo Alto, California; Division of Retrovirology, Walter Reed Army Institute of Research, and Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, and Virus Biology Section, Laboratory of Tumor Cell Biology, National Cancer Institute, Frederick, Maryland

Six procedures for quantifying plasma human immunodeficiency virus type 1 (HIV-1) RNA were evaluated by nine laboratories. The procedures differed in their sample volume and preparation of samples and methods of amplification and detection. Coded samples in a 10-fold dilution series of HIV-1–spiked plasma were correctly ranked by all six procedures. Subsequently, coded duplicate plasma samples from 16 HIV-1–infected patients were tested using a common set of standards. Several HIV-1 RNA procedures were sufficiently reproducible so that an empiric 4-fold change could be viewed as significant. HIV-1 RNA levels in the patients (up to 370,000 RNA copies/mL) correlated with proviral HIV-1 DNA and were inversely correlated with CD4 cell counts; HIV-1 RNA assays were more sensitive than plasma viremia, standard p24 antigen, or immune complex–dissociated p24 antigen assays. This study demonstrated that several HIV-1 RNA quantitative assays are ready for use in clinical trials.

A number of immunologic and virologic surrogate markers have been used in clinical trials for the treatment of human immunodeficiency virus type 1 (HIV-1) to predict clinical response to therapy [1]. These markers, which have all been directly implicated in the disease process, include absolute CD4 cell count, serum HIV-1 p24 antigen level with and without immune complex dissociation (ICD), and quantitative HIV-1 microculture (QMC) of peripheral blood mononuclear cells (PBMC) or plasma.

The application of virologic markers in clinical studies was recently reviewed [2]. Experience with such markers has revealed limitations. In a large clinical trial [3], changes in absolute CD4 cell count did not correlate well with clinical response to antiretroviral therapy. Assays for serum HIV-1 p24 antigen levels have limited sensitivity: They are positive in only 26% of patients with absolute CD4 cell counts of 200–500/μL and in 69% with counts <200/μL [4]. Even with the improved sensitivity after an ICD step, HIV-1 p24 antigen can be detected in the serum of only half of the asymptomatic infected patients [5]. Likewise, QMC for the measurement of HIV-1 in plasma has limited sensitivity, being positive in only 23% of asymptomatic infected patients [6], and it has an inherent variation of 1.2 $\log_{10}$ [7]. QMC for the measurement of HIV-1 in PBMC is more sensitive but also has a high degree of variability.

A number of assays using either polymerase chain reaction (PCR) or branched DNA (bDNA) signal amplification technology [8] have recently been developed for measuring HIV-1 RNA in plasma of infected persons. A relationship be-

Received 17 November 1993; revised 4 April 1994.

Presented in part: IXth International Conference on AIDS, 7 June 1993, Berlin.

Informed consent was obtained from subjects, and human experimentation guidelines of the US Department of Health and Human Services or those of the authors' institutions were followed in the conduct of this clinical research.

The following authors have a commercial interest that could pose a potential conflict of interest: Belinda Yen-Lieberman (site C4) owns stock in Chiron and did the coded panel with the Chiron assay. Robert Kokka (site C6) is an employee of and owns stock in Chiron and did the coded panel with the Chiron assay. Denis Henrard (site A1) is an employee of and owns stock in Abbott Laboratories and did the coded panel with the Abbott procedure. Shirley Kwok (site D) is an employee of Roche Molecular Systems and did the coded panel with the Roche procedure, and she also owns stock in Chiron.

Financial support: National Institutes of Health (AI-82517 to Baylor College of Medicine) and cooperative agreements with Case Western Reserve University (AI-25879), University of Washington (AI-27664), University of Southern California (AI-27673), and Stanford University (AI-27666); Roche Molecular Systems; Abbott Laboratories; and Chiron.

Reprints or correspondence: Dr. J. Brooks Jackson, Institute of Pathology, Case Western Reserve University, 2085 Adelbert Rd., Cleveland, OH 44106.

The Journal of Infectious Diseases 1994;170:553–62

0022-1899/94/7003-0008$01.00

tween HIV-1 RNA plasma levels and stage of disease and clinical progression has been found with these assays [9–11]. Moreover, with these assays, several investigators have shown decreased plasma HIV-1 RNA levels in patients starting antiretroviral therapies [11–16]. One study that used a combination antiretroviral therapy showed that median changes in CD4 cell counts were significantly greater in patients who had >1 log decrease in plasma HIV-1 RNA titer than in patients with no change [15]. In addition, several of the published methods are sufficiently sensitive to detect and quantify plasma HIV-1 RNA in >96% of HIV-1–seropositive patients, including asymptomatic patients [10, 14, 16].

These preliminary studies indicate that one or more of these quantitative HIV-1 RNA assays will probably be useful in studying the pathogenesis of HIV-1 infection and in assessing the effect of antiretroviral drugs and immunotherapies. Therefore, a comparative study was done to evaluate six HIV-1 RNA detection assays in terms of their abilities to detect 10-fold changes in plasma HIV-1 RNA concentration, to obtain preliminary data on the sensitivity, specificity, and reproducibilty in a group of patients with a wide range of absolute CD4 cell counts, and to compare plasma HIV-1 RNA measurements with other virologic markers (direct and ICD HIV-1 p24 antigen, QMC of PBMC and plasma, and HIV-1 DNA copies/10^6 CD4 cells).

Materials and Methods

Preparation of HIV-1 virus stock. Human plasma spiked with HIV-1 was prepared at the Virology Reference Laboratory (VRL), Baylor College of Medicine, as follows. Anti–HIV-1–negative plasma, collected in citrate-phosphate-dextrose anticoagulant, was obtained from the Gulf Coast Regional Blood Center (Houston). HIV-1 stock was prepared by coculturing PBMC from an HIV-1–infected pediatric patient with seronegative donor PBMC. After 11 days in culture, the supernatant fluid was separated from the cells by centrifugation at 2800 *g* for 10 min at 22°C, diluted with an equal volume of anti–HIV-1–negative plasma, aliquoted, and stored in the vapor phase of liquid nitrogen at −180°C. Sets of reference standards were prepared subsequently by serial 10-fold dilutions of this virus stock with anti–HIV-1–negative human plasma, then stored in liquid nitrogen. Although DNA is reported to be present in HIV-1 particles [17, 18], no proviral HIV-1 DNA was detectable in the virus stock, as shown by experiments comparing the degree of amplification obtained with and without prior incubation with reverse transcriptase.

Estimates of HIV particle concentration. The concentration of HIV-1 in the virus stock was quantified by two methods at the Virus Biology Section, Laboratory of Tumor Cell Biology, National Cancer Institute [19]. Polystyrene beads (149.0 ± 4.0 nm; Duke Scientific, Palo Alto, CA) were added to an aliquot of the virus stock to obtain a concentration of 10^9 beads/mL. The resulting stock was ultracentrifuged (155,000 *g* for 40 min at 20°C), and the pellet was fixed in 2.5% glutaraldehyde prior to embedding in epoxy resin (LX112; Ladd Research, Williston, VT) for analysis by electron microscopy. The ratio of spheres to virions was evaluated, and the particle density was determined. An independent estimate of virus concentration was obtained by centrifuging the HIV-1 virus stock, assaying the p24 antigen in the pellet, and dividing the result by 5×10^{-17} g of p24 core protein per particle, the factor being based on the molecular weight of the protein, which was calculated from its amino acid sequence [19].

Preparation of plasma panels and processing of blood. Of 20 volunteer donors, 4 were known to be negative for HIV-1 antibody and 16 were HIV-1–positive by Western blot. Eight of the HIV-1–positive donors were selected because their CD4 cell counts were >450/μL (range, 494–1522); the other 8 had counts <250/μL (range, 12–234).

Blood (120 mL) was collected from each subject in tubes containing acid-citrate-dextrose and processed within 6 h. A sample was sent immediately for CD4 and differential white blood cell counts and for assaying for other T cell markers. Plasma was separated from whole blood by centrifugation. Most plasma was used to prepare several panels of 40 samples each, using coded duplicates from the 20 volunteers; the remaining plasma was aliquoted and stored in liquid nitrogen for subsequent virologic testing. PBMC were separated from diluted whole blood in a ficoll-hypaque gradient, and a portion (5-fold serial dilutions starting with 10^6 PBMC) was tested for the presence of HIV-1 p24 antigen in a QMC assay. The remaining PBMC were cryopreserved in liquid nitrogen at 5×10^6 cells/mL, while aliquots of 2×10^6 PBMC were stored as liquid-free pellets for HIV-1 DNA PCR testing.

Test panels. Two coded panels were prepared. The first, consisting of 9 samples, comprised a single sample each of a negative plasma, a 10-fold (10^7 copies/mL) and a 10^5-fold (10^3 copies/mL) dilution of the virus stock, plus duplicate samples of 10^2-, 10^3-, and 10^4-fold dilutions of the virus stock (10^6 copies/mL, 10^5 copies/mL, and 10^4 copies/mL, respectively). The assignment of nominal HIV-1 RNA copies per milliliter is described below. The second panel comprised 40 plasma samples (duplicates from 20 donors).

Standards. The same set of standards was used at all sites in testing the second panel, with the objective of improving comparability among test results produced at different sites. The standards corresponded to the samples in the first panel, except that virus stock dilutions ranged from 10^2- to 10^6-fold (10^6–10^2 copies/mL). We used this material because it had been quantified at five sites.

The median value of the measurements made on the 10-fold dilution of virus stock was 1.4×10^7 RNA copies/mL; this was rounded to one significant figure, giving the value of 10^8 RNA copies/mL of virus stock, which agreed reasonably well with the two independent estimation methods described above. By electron microscopy, 1.61×10^8 HIV-1 particles/mL of virus stock were measured, and 1.68×10^8 particles/mL were estimated by p24 antigen assay in a pellet obtained by ultracentrifugation of the virus stock.

An estimate of the number of HIV-1 RNA copies per milliliter based on multiplying the particle concentration by 2 could

not be made with any certainty. The presence of "empty" particles containing <2 RNA strands or the occurrence of genomes with variant sequences that failed to anneal to the primers or probes would reduce the estimate of RNA copy number based on virus particle number. Therefore, we assigned a nominal concentration of 10^8 RNA copies/mL to the virus stock as a rough estimate. The VRL standards, made by serial 10-fold dilutions from the stock, ranged from 10^6 to 10^2 copies/mL.

HIV-1 p24 antigen testing (Coulter, Miami) of these standards showed the 10^5-copy/mL standard to be the highest dilution with detectable p24 antigen (8 pg/mL). This figure is in close agreement with the estimate of 10^4 HIV-1 RNA copies/pg of p24 antigen reported by Piatak et al. [10].

Standard tests. All virologic tests were done under code. Absolute CD4 cell counts were obtained by flow cytometry (Profile analyzer; EPICS, Division of Coulter Electronics, Hialeah, FL). Plasma viremia, QMC, HIV-1 proviral DNA assays on PBMC, and direct and ICD p24 antigen assays (Abbott Laboratories) were done at VRL using standard procedures [20–22]. HIV-1 proviral DNA was quantified in pellets consisting of 2×10^6 PBMC prepared with ficoll-hypaque gradients [21], using the Gen-Probe (San Diego) detection method [23]. Where necessary, dilutions of the cell lysates were made before amplification to obtain instrument responses that fell within the range displayed by HIV-1 DNA copy number standards (0, 2, 5, 10, 20, 50 copies/amplification). The results were expressed as HIV-1 DNA copies per 10^6 CD4 cells, based on the monocyte, lymphocyte, and CD4 cell counts for the blood sample.

Quantification of HIV-1 RNA in plasma. Eleven sites using eight procedures participated in the project. Two sites voluntarily withdrew because of other commitments or technical problems. Thus, results from nine sites using six different procedures are reported. Procedures D and E, respectively, are described in full elsewhere [16, 24]. Table 1 summarizes the essential features of the six procedures. Sample requirements ranged from 100 to 2000 μL. In procedure A, the virus was concentrated by immunocapture [25], and in C, E, and F, it was pelleted by high-speed centrifugation; no attempt was made to concentrate the virus in procedures B and D. Lysis of the virus was accomplished in procedures A and C with reagents developed at Abbott Laboratories and Chiron, respectively. Lysis was achieved with a guanidinium reagent [26] followed by precipitation of RNA with alcohol in procedures B–F.

Amplification was done by reverse transcription–PCR in all procedures except C, in which the signal rather than the genomic target sequence was amplified. Procedures A, B, and E used the same primers (SK38 and SK39), while different primer pairs were used in D (SK 462/431) and F (*gag* 1547/1412).

Detection methods also differed. In procedure A, visual comparison of autoradiographs was used [25]. The Gen-Probe detection method, which is based on chemoluminescence of acridinium esters [23], was used in procedure B. In C, signal amplification, wherein viral nucleic acid is captured via oligonucleotide probes complementary to conserved regions of the HIV-1 *pol* gene, was used. bDNA amplifier molecules were then hybridized to the immobilized target-probe complexes, followed by hybridization of multiple alkaline phosphatase–labeled probes to each bDNA molecule [8]. Complexes were detected by hydrolysis of a chemoluminescent dioxetane substrate. Procedures D and E used detection methods based on changes in optimal density produced by reactions mediated by horseradish peroxidase. Procedure F used phosphorimaging for quantification of the PCR product after hybridization to an isotopically labeled probe.

Table 1. Site procedures for the quantification of HIV-1 RNA.

Procedure, sample volume (μL)	Description	Name (no.) of site using procedure
A, 100	Immunocapture and lysis; RT-PCR with SK38, SK39 primers; liquid hybridization; visual comparison of autoradiographs; result based on 2 observations	Abbott (1), University of Southern California (2), University of Washington (3), Cleveland Clinic Foundation (4)
B, 200	Guanidinium-phenol; alcohol precipitation; RT-PCR with SK38, SK39; detection with Gen-Probe kit; quantification based on chemoluminescence; result based on 4 observations	Baylor College of Medicine (5)
C, 2000	Pelleting at 23,500 *g* for 1 h at 4°C; lysis; amplification with alkaline phosphatase linked to probes specific for *pol* gene; quantification based on chemoluminescence; result based on 2 observations	University of Washington (3), Cleveland Clinic Foundation (4), Chiron (6)
D, 200	Guanidinium; alcohol precipitation; single-enzyme RT-PCR with SK431, SK462 primers; enzyme-linked assay; detection based on OD; internal quantification standard used; results based on 4 observations	Roche Molecular Systems (7)
E, 450	Pelleting at 100,000 *g* for 15 min at 10°C; guanidinium-phenol; alcohol precipitation; RT-PCR with SK38, SK39 primers; enzyme-linked assay; detection based on OD; results based on 2 observations	Stanford University (8)
F, 500	Pelleted at 118,000 *g* for 1 h at 4°C; lysis; RT-PCR with *gag* 1547, *gag* 1412 primers; liquid hybridization; detection by phosphorimaging; results based on single observation	Walter Reed Army Institute of Research (9)

NOTE. RT-PCR, reverse transcription and polymerase chain reaction; OD, optical density.

Each procedure used different standards. Procedure A used HIV-1–seronegative plasma spiked with virus from tissue-culture supernatant and purified HIV-1 RNA. Procedure B used DNA copy number standards prepared from 8E5/LAV (lymphadenopathy-associated virus) cells [27]. Procedure C used a single-stranded bacteriophage DNA containing an HIV-1 *pol* gene–cloned insert, which was calibrated against a highly characterized HIV-1 RNA transcript. RNA transcripts were used in procedures D, E, and F. Site 3 also used DNA standards prepared on site from ACH-2 cells. These standards were used in testing both panels, as was the common set of standards.

Statistical analysis. Basic descriptive statistical analyses were done, including tabulation of method sensitivities and specificities, reproducibility (expressed as standard deviations [SD]) of log copy estimates pooled over individuals, and correlations among RNA levels and other surrogate markers. Estimates of reproducibility were used to calculate error rates associated with the decision of whether 2 samples have different copy numbers, assuming the SD estimates are correct and the methods are unbiased. Analysis of variance (ANOVA) based on ranks was used to test for overall method effects, and *t* tests based on ranks were used to compare individual methods. All correlations were preceded by log transformations of variables.

The median VRL HIV-1 RNA copies per millimeter (shown in table 2) were calculated as follows. For each of the six procedures, the median result for all sites using that procedure was calculated. The median of these six procedure medians was used as the nominal VRL copies per milliliter for each patient.

The reproducibility of each method was estimated from data on the second panel, which consisted of 20 pairs of encoded duplicate samples from the 20 volunteers. With most procedures, two or four observations were made on each sample to obtain a result (table 1). The average estimated result, based on VRL standards, was calculated for each of the duplicate samples and converted to a logarithm. The SD of the two log averages was calculated using only sample pairs where both duplicates were positive; this value was called the duplicate SD. (An outlying pair was omitted from the results from site 5.) The reproducibility for each procedure was expressed by the duplicate SD pooled over the pairs with both duplicates positive. Procedure A was omitted from these calculations because of its semiquantitative nature.

These estimates of reproducibility were used to formulate decision rules for comparing samples and to estimate error rates associated with such rules. Derivation of K-fold change rules (where K may be any number) and their error rates for comparison of positive specimens assumes that log copy estimates for distinct samples are independently normally distributed with constant additive bias and constant SD.

Results

Detection of HIV-1 RNA in a dilution series. The first of the two panels that were tested consisted of a 10-fold dilution series prepared with seronegative plasma spiked with an HIV-1 culture supernatant. Researchers at each site were asked to identify the sample with the highest level of HIV-1 RNA, which all did correctly. Negative samples were correctly reported as having no detectable HIV-1 RNA. Participants were also asked to rank, in order, the 7 other samples below the highest sample. At five sites using different procedures, RNA concentrations were measured in HIV-1 RNA copies per milliliter (figure 1A) and samples were ranked correctly. Even though results were based on the standards made at each site and were composed of different materials, there was close agreement among them in the sample with the highest concentrations of virus. The results revealed more interlaboratory variation for the 10^4- and 10^5-fold dilutions (10^4 and 10^3 copies/mL). Figure 1B summarizes results obtained with procedure A at three sites that correctly ranked the 3 most concentrated samples. Here again, variability was observed at the higher dilutions; one site was unable to detect RNA in the 10^5-fold dilution (10^3 copies/mL).

Testing clinical specimens. The second panel consisted of plasma specimens from patients with a wide range of CD4 cell counts. Table 2 summarizes the results obtained using the VRL standards to estimate the HIV-1 RNA concentrations. Results for each of the blinded duplicate samples are given to show the test reproducibilty at each site and to indicate the range of values observed. Results, arranged in order of increasing HIV-1 RNA copies per milliliter for the 4 seronegative donors (A-D) appear first, followed by those for the 16 seropositive patients. Since the actual HIV-1 RNA concentrations were unknown, median values were calculated for 15 of the 16 donor plasma samples (in donor P, the HIV-1 RNA level was barely detectable or undetectable). Estimates ranged from 1500 to 366,300 copies/mL in the 15 anti-HIV–positive patients, whose CD4 cell counts ranged from a high of 1522 to a low of 12 CD4 cells/μL.

Table 3 summarizes data in table 2 by showing the results from procedures used at the different sites. Results for donor P were not included because HIV-1 RNA was detected in only 1 of the 2 duplicate samples using procedure B, and inhibition was reported in the assays of both samples of this plasma specimen using procedure D. The results on the 19 other donors fell into three groups: HIV-1–negative (4 samples), low-positive with nominal values between 10^3 and 10^4 copies/mL (5), and high-positive with $>10^4$ copies/mL (10). Concordant results in the duplicates established a sample as positive or negative; discordant results were designated indeterminate. If 1 of the 2 duplicate samples was indeterminate, the result of the other duplicate determined its grouping.

It appears with this relatively small number of samples that all six methods were capable of correctly discriminating between samples from anti–HIV-1–negative donors and samples with relatively high concentrations of HIV-1 RNA. The methods differed in their false-negative rates on the low-positive samples. These observations were in line with results shown in figure 1, where the accuracy of the procedures de-

Table 2. Quantification of HIV-1 RNA in duplicate samples of anti-HIV–negative and anti-HIV–positive plasma specimens based on VRL reference standards.

Category, donor	CD4 cells/μL	Median 10^3 RNA copies/mL	HIV-1 RNA copies/mL (× 1000) by procedure and site*									
			A1	A2	A4	B5	C4	C6	C3	D7	E8	F9
Anti-HIV–negative												
C	828	0	ND	ND	ND	ND	IND	<CO	<CO	ND	<CO	<CO
			ND	ND	ND	ND	<CO	<CO	<CO	ND	<CO	<CO
B	856	0	ND	ND	ND	ND	<CO	<CO	<CO	ND	<CO	<CO
			ND	ND	ND	ND	<CO	<CO	<CO	ND	<CO	<CO
A	1321	0	ND	ND	ND	ND	<CO	<CO	<CO	ND	<CO	<CO
			ND	ND	ND	ND	<CO	<CO	<CO	ND	<CO	<CO
D	1475	0	ND	ND	ND	ND	<CO	<CO	IND	ND	<CO	<CO
			ND	ND	ND	ND	<CO	<CO	<CO	ND	<CO	<CO
Anti-HIV–positive												
P	1382	†	ND	ND	ND	ND	<CO	<CO	IND	—	<CO	<CO
			ND	ND	ND	0.8	<CO	<CO	<CO	—	<CO	<CO
M	706	1.5	1.0	0.1	ND	1.6	<CO	<CO	<CO	2.3	<CO	<CO
			1.0	1.0	100.0	1.9	<CO	<CO	<CO	2.4	1.4	<CO
W	494	1.7	1.0	0.1	ND	2.1	<CO	<CO	<CO	3.4	1.3	2.3
			1.0	100.0	ND	2.7	IND	<CO	IND	3.0	<CO	4.3
X	715	2.5	1.0	0.1	100.0	0.3	<CO	<CO	11.0	5.5	3.9	2.3
			1.0	5.5	ND	2.1	<CO	<CO	IND	8.6	<CO	4.8
E	74	5.7	1.0	5.5	100.0	5.9	10.7	<CO	13.8	9.7	3.2	8.8
			1.0	3.0	100.0	6.8	10.0	IND	<CO	5.9	2.6	14.2
U	841	8.8	1.0	10.0	<CO	14.5	IND	<CO	IND	12.9	6.3	8.9
			1.0	0.1	100.0	4.9	10.9	7.7	8.5	9.1	9.5	24.6
Z	515	11.5	10.0	10.0	100.0	13.5	IND	11.4	<CO	19.7	2.7	58.9
			10.0	10.0	100.0	9.7	12.7	13.5	15.8	14.3	13.5	59.2
K	165	18.2	10.0	1.0	100.0	17.0	18.4	16.2	22.3	12.2	22.9	14.5
			10.0	10.0	100.0	26.5	20.9	13.2	16.4	12.4	15.5	20.0
V	1522	22.8	NT	NT	NT	10.9	22.7	19.5	15.2	20.5	10.2	91.5
			10.0	32.5	100.0	3.1	18.0	27.2	IND	30.1	17.9	141.8
I	27	39.2	10.0	0.1	100.0	13.7	48.6	43.0	33.8	89.5	38.0	46.2
			100.0	0.3	100.0	9.7	37.7	43.7	26.6	92.6	32.4	24.1
J	234	50.0	10.0	10.0	100.0	38.5	59.6	81.4	30.9	93.5	43.7	407.9
			10.0	0.1	100.0	34.0	59.7	54.6	51.0	82.8	37.2	87.1
Y	723	66.4	100.0	7.5	100.0	20.5	66.6	50.7	64.7	76.4	21.9	129.3
			10.0	ND	100.0	9.4	61.8	71.5	76.7	60.8	16.2	138.4
F	46	137.2	100.0	1.0	100.0	29.5	145.2	107.2	173.2	146.5	40.7	1647.9
			10.0	550.0	1000.0	90.0	179.5	122.8	89.7	115.6	100.0	1654.8
S	12	139.9	10.0	55.0	100.0	540.0	103.3	101.1	82.9	183.0	47.9	355.5
			100.0	0.1	1000.0	76.0	91.0	101.1	56.4	182.5	50.1	447.7
O	14	278.7	100.0	5.5	100.0	65.5	487.7	718.8	396.4	513.2	43.7	1105.6
			100.0	1000.0	100.0	68.5	576.9	572.2	327.7	401.6	63.1	426.1
N	47	366.3	100.0	1.0	100.0	44.5	323.8	289.6	290.4	477.9	100.0	494.9
			100.0	1.0	100.0	1575.0	280.4	290.7	353.3	383.1	69.2	1475.6

NOTE. ND, not detected (zero instrument response); <CO, below a predetermined cutoff value; —, inhibition; NT, not tested; IND, indeterminate.

* Identified in table 1.

† Results not included because HIV-1 RNA was detected in only 1 of 2 duplicate samples using procedure B, and inhibition was reported in assays of both samples using D.

creased in testing the 10^4- to 10^5-fold dilutions that corresponded, respectively, to 10^4 and 10^3 nominal copies/mL. It is possible that some of the variability was due to lack of experience with a given procedure at some sites. Thus, for further analyses of results, we used data from the commercial manufacturers as representative of their methods. Therefore, methods done at Abbott and Chiron were statistically analyzed using the data in table 2 (columns A1 and C6, respectively).

Reproducibility and method differences. The reproducibility of each procedure was expressed as the duplicate SD (S) of log copies, pooled over sample pairs where both duplicates

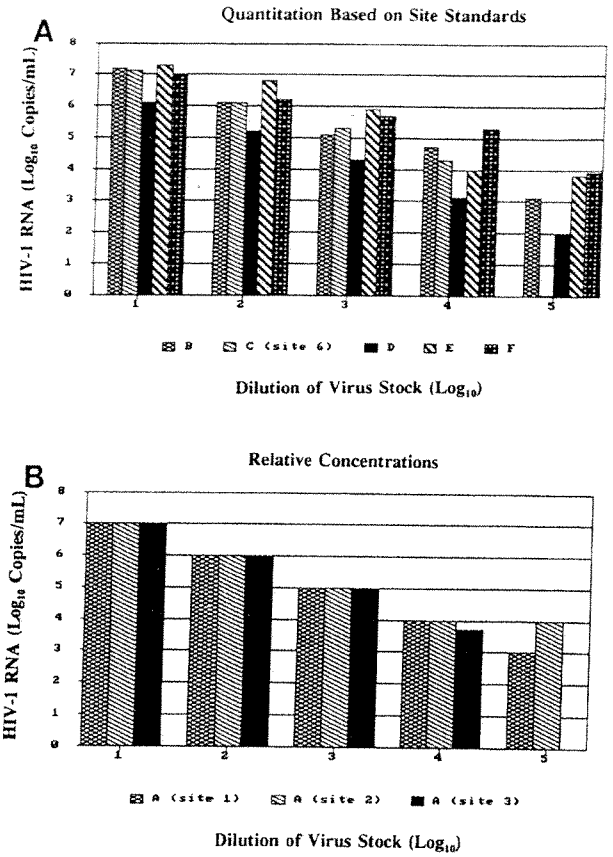

Figure 1. HIV-1 RNA levels in dilution series prepared from spiked plasma. Keys refer to procedures and sites in table 1. **A**, Concentration levels based on site standards. **B**, Relative concentrations ranked by visual comparison of autoradiographs. Sample with highest HIV-1 RNA concentration was given rank of 1; others were ranked below it (i.e., 10^{-1}, 10^{-2}). For comparison with **A**, rank values were transformed by formula $y = 7 + \log_{10}$.

were positive. These S values were 0.674 (procedure B), 0.163 (C), 0.188 (D), 0.433 (E), and 0.517 (F). Thus, four procedures had estimated pooled SDs ≤0.52. A method or laboratory with true S = 0.50 can discriminate true 10-fold differences in copy numbers. That is, for repeated analyses of the same sample (or 2 samples with the same number of copies), 95% of the estimated copy number ratios are expected to lie between 0.25 and 4.0. Hence, if analyses of 2 samples produce a ratio outside this interval, it is implausible that the samples have the same concentration, and the hypothesis of no difference between them is rejected at the 5% significance level. This 4-fold decision rule has .90 power against true 10-fold changes (i.e., if 2 samples truly differ ≥10-fold, then the probability is at least .90 that the 4-fold rule will lead to the correct decision that they are different). For a laboratory with S = 0.25, a ≥2-fold difference is significant at the 5% level; this 2-fold rule has power .87 against true 3-fold changes.

VRL standards were compared to site standards while testing the second panel. The dose-response curves for the two sets were nearly coincident using procedures C, E, and F (data not shown). In contrast, the standard curve obtained using procedure D was shifted slightly to the left of the VRL standard curve, indicating some disagreement in the assigned nominal values. In such circumstances, a common reference standard can be useful in aligning different measurement methods. However, even with the use of common standards, it appears that method differences existed. Methods effects were investigated using ANOVA of ranks, with pairwise comparison of methods by ordinary *t* tests based on the ranks. The basic data for these method comparisons were the overall averages of the results at each site for each of the 10 patients with $\geq 10^4$ nominal RNA copies/mL. These analyses were done without the data obtained with procedure B, which were somewhat more variable than those from other methods. The difference in overall method effect was highly significant ($P < .0001$) irrespective of the standards used for calculations.

Relation of plasma HIV-1 RNA to other measures of HIV-1 infection. In table 4 the concentration of HIV-1 RNA in sera from the 16 patients is related to results from other quantitative tests for HIV-1 infection. There was an inverse correlation between HIV-1 RNA and CD4 cell counts ($r = -.71$; $P < .05$) and a positive correlation between HIV-1 RNA and HIV-1 DNA ($r = .80$; $P < .05$). The correlation between HIV-1 DNA (expressed as copies/10^6 CD4 cells) and CD4 cell count was significant ($r = -.83$; $P < .05$). The correlation between HIV-1 RNA and QMC was relatively weak but statistically significant ($r = .52$; $P < .05$). The correlations between QMC and HIV-1 DNA (r = .41) and between QMC and CD4 cell counts (r = −.19) were statistically insignificant ($P > .05$).

Among the 16 patients there was at least a 100-fold variation between the highest and lowest measurable values found in assays for CD4 cell counts, HIV-1 RNA, proviral HIV-1 DNA, and QMC. These four tests gave quantitative information for 15 of the 16 patients. In contrast, the plasma viremia assay was positive in only 8. The remaining two tests were also relatively insensitive as markers for HIV-1 infection: The direct and the ICD p24 antigen assays were positive in 4 and 6 patients, respectively. Moreover, none of the 10 patients with CD4 cell counts >100/μL were positive by the direct p24 antigen assay, and only 1 was by the ICD p24 antigen assay. All patient samples negative by the ICD p24 antigen assay had $\leq 5 \times 10^4$ HIV-1 RNA copies/mL, which is consistent with the approximate p24 antigen detection limit of 8 pg/10^5 HIV-1 RNA copies/mL in testing the standards.

Discussion

Correlation of plasma HIV-1 RNA to other tests. Some interesting observations can be made. First, a strong correlation exists between the CD4 cell counts and the levels of

Table 3. Results of 6 procedures for estimation of HIV-1 RNA in plasma.

Procedure and site*	4 negative specimens	5 specimens with 10^3–10^4 HIV-1 RNA copies/mL			10 specimens with $>10^4$ HIV-1 RNA copies/mL		
	Negative	Positive	Indeterminate†	Negative	Positive	Indeterminate	Negative
A1	4	5	0	0	9‡	0	0
A2	4	5	0	0	8‡	1	0
A4	4	1	3	1	9‡	0	0
B5	4	5	0	0	10	0	0
C4	4	2	0	3	10	0	0
C6	4	0	1	4	10	0	0
C3	4	2	1	2	10	0	0
D7	4	5	0	0	10	0	0
E8	4	2	3	0	10	0	0
F9	4	4	1	0	10	0	0

NOTE. Results from donor P are omitted from this table. Data represent results on duplicate samples.
* Identified in table 1.
† Discordant results on duplicate samples.
‡ No duplicate sample for 1 specimen.

cellular HIV-1 DNA and plasma HIV-1 RNA detected. Second, among the 5 samples collected from patients with CD4 cell counts $<50/\mu L$, all virologic assays yielded positive responses, and 6 of the 8 samples that were positive in the plasma viremia assay came from patients with CD4 cell counts $<100/\mu L$. Finally, samples with HIV-1 RNA concentrations $>5 \times 10^4$ copies/mL were likely to be positive by the plasma viremia and the ICD p24 antigen assays.

Use of HIV-1 RNA assays in clinical trials. The broad range of HIV-1 RNA concentrations that we found in HIV-1–infected patients is consistent with previously reported ranges (from undetectable to $>10^7$ RNA copies/mL, depending on the stage of disease) [9–12]. The current study showed that amplification-based procedures for measuring HIV-1 RNA extend our ability to more sensitively assess clinical samples. Previous studies have suggested using

Table 4. Virologic markers in relation to decreasing CD4 cell counts.

CD4 cells/μL*	HIV-1 RNA† (copies/mL)	HIV-1 DNA (copies/10^6 cells)	Quantitative microculture (IU/10^6 cells)	Plasma viremia (IU/mL)	p24 antigen (pg/mL)	
					Direct	ICD
1522	22,800	1300	206	ND	ND	ND
1382	—	100	ND	ND	ND	ND
841	8800	2200	82	ND	ND	ND
723	66,400	1200	206	1.6	ND	42
715	2500	550	16	ND	ND	ND
706	1500	2200	8.7	1.6	ND	ND
515	11,500	15,000	16	ND	ND	ND
494	1700	870	0.5	ND	ND	ND
234	50,000	3700	16	ND	ND	ND
165	18,200	8200	1.6	ND	ND	ND
74	5700	8300	16	41	ND	ND
47	366,300	28,000	2.5	206	52	36
46	137,200	35,000	421	3.2	ND	33
27	39,200	4700	1.6	16	20	60
14	278,700	54,000	82	>14500	156	264
12	139,900	58,000	421	82	22	30

NOTE. Samples from 4 anti-HIV–negative donors were nonreactive in HIV RNA, DNA, and p24 antigen assays (data not shown). IU, infectious units; ICD, immune complex dissociated; ND, not detected.
* Reference range, 561–1405 cells/μL.
† Median value of estimates based on Virology Reference Laboratory standards using all 6 procedures. No estimate could be made on results from donor P (CD4 cell count, 1382/μL).

plasma HIV-1 RNA assays to define the status of HIV-1 infection [9–11]. Plasma HIV-1 RNA levels may respond quickly to some drug therapy regimens [10, 11, 13, 16]; the same may not be true of HIV-1 DNA that is integrated into the host genome [13, 14]. Moreover, CD4 cell counts may remain relatively unchanged following treatment with drugs that appreciably lower plasma HIV-1 RNA levels [12, 13].

Although HIV-1 RNA measurement may prove to be useful in assessing drug efficacy in clinical trials, the importance of the other tests should not be overlooked since they give different kinds of information [2]. The CD4 cell count may reflect the extent of damage to the immune system; however, the effects of other factors leading to the sequestration of cells or a decrease in recirculation from lymph nodes to blood are unknown. Plasma HIV-1 RNA may represent virus recently produced and exported. Proviral HIV-1 DNA in CD4-positive cells represents integration of the viral genome into the host cell, which is believed to be an early event in HIV-1 infection. QMC and plasma viremia are tests for infectivity in vitro. p24 antigen assays detect HIV-1 capsid protein, which may or may not be virion-associated. The combined information, content, and relative importance of these tests can only be determined from their assessment in the same set of patients in controlled clinical trials.

Approaches to measurement of plasma HIV-1 RNA. It is interesting that widely divergent methods of detection, such as phosphorimaging, bDNA technology, and visual inspection of autoradiographs, can produce similar results. Thus, several approaches are available to investigators for measuring changes in plasma HIV-1 RNA. A number of assay factors, apart from specificity, sensitivity, and precision, must be considered when deciding which methods to use in clinical trials (e.g., sample volume, throughput, cost, ease of performance, and turn-around time). The results from our study are not sufficient to choose one method over another. Furthermore, some of the procedures used in this study were in early development and, therefore, do not represent final products. Since it is possible that all of the procedures evaluated may have specific clinical applications, their advantages should be highlighted.

The immunocapture methods used in procedure A may offer an alternative to the more time-consuming guanidinium-extraction method. Procedures B and D were sensitive methods, as shown by their ability to detect 10 or fewer copies of HIV-1 DNA [23], but B displayed the largest variation. Procedure C, on the other hand, had a very broad working range from its provisional cutoff level of 10^4 to 1.6×10^6 nominal RNA copies/mL, and reproducibility with C and D was among the highest. D and F are the only procedures that incorporate dUTP and uracil-*N*-glycosylase for carryover prevention [28]. In addition, D uses a single enzyme for both reverse transcription and PCR reactions, which are done uninterrupted in a single tube. Procedure E has proven its usefulness in a number of studies involving large numbers of specimens [9, 12, 16]. Procedure F uses phosphorimaging to detect an isotopic signal. The advantages of this technology compared with conventional exposures of radiographic film include significantly reduced exposure times (minutes not days) and linear sensitivity and dynamic ranges that are 100 and 400 times greater, respectively, than those of film. In addition, all data generated, including the gel image and band intensity, are archived on tape [29].

ANOVA based on ranks showed differences between methods, suggesting the possibility that real systematic differences exist. There are many plausible explanations for such biases, including complementarity of the primers and probes with RNA in particular samples, variation in the amount of virus pelleted by high-speed centrifugation and in the amount of HIV-1 RNA recovered during extraction, and use of different assay diluents. However, it should be realized that site and method are confounded in our analysis. The conclusion of systematic differences among methods is a tentative one, which should be investigated further. Until this issue is settled, it seems reasonable to require that the same RNA assay be specified for a given clinical trial. Ideally, the issue should be resolved by using multiple methods on all samples in a clinical trial.

The main objectives of this study were to detect 10-fold changes in plasma HIV-1 RNA concentration and, to a lesser extent, to evaluate the specificity and sensitivity of the available quantitative HIV-1 RNA assays. We applied a 4-fold decision rule, whereby 2 samples were regarded as significantly different if the ratio of maximum to minimum estimated copy numbers exceeds four, assuming a duplicate SD ≤ 0.5. Against a true 10-fold difference, the 4-fold rule theoretically produces a correct decision at least 90% of the time. In fact, 10-fold differences were designed into the first panel, and duplicate samples were present at three concentrations.

Quantitative analysis of results of the first panel with procedures B-F showed that 51 (85%) of 60 possible within-site comparisons between samples that truly differed 10-fold were correctly classified using this 4-fold rule; procedure A was excluded from analysis because of its semiquantitative nature. Furthermore, 14 (93%) of 15 within-site comparisons between duplicates were correctly classified as having the same number of copies. Empirical error rates calculated from the first panel therefore show good agreement with the theoretical error rates (15% vs. 10% and 7% vs. 5%, respectively). However, it is important to note that the validity of any K-fold–change rule is predicated on knowledge of the true SD of a particular assay within a given laboratory. Therefore, each laboratory should use a quality control program designed to produce information needed to estimate the true SD in order to validate a given K-fold–change rule.

While we have preliminary information on the reproducibility of different methods, no recommendations about the

selection of specific HIV-1 RNA assays can be made. More information on real-time accuracy and precision can be obtained by incorporating blind splitting of patient samples and appropriate positive controls with every batch of unknown samples. In addition to using a common RNA standard for quantification, a common set of positive and negative control materials should be used by all laboratories determining HIV-1 RNA levels for multicenter clinical trials. This would allow for the identification of systematic differences among laboratories, comparison of the analytic precision of different assay methods, and monitoring for amplicon contamination.

Clinical trials are currently testing several of these methods for quantifying HIV-1 RNA to determine if relative changes in HIV-1 RNA are useful in predicting clinical response to therapy and progression to disease. Clearly, much work remains to be done before the significance of plasma HIV-1 RNA levels is understood. Apart from their use in clinical trials, further studies should be done on the utility of these assays in different clinical settings. At the same time, the issues of standardization and differences among methods should be addressed before these assays are applied to routine clinical practice.

Acknowledgments

We thank the following for excellent technical assistance: Jack Phillips (Abbott Diagnostics); Michail Haywood, Edward Seidel, and Garrett Stephens (Baylor College of Medicine); Brad Hoo (Chiron); Colleen Starkey (Cleveland Clinic Foundation); Daniella Livnat (National Institute of Allergy and Infectious Diseases); Cindy Christopherson, Nancy McKinney, and John Mulder (Roche Molecular Systems); Zhiliang Li (University of Southern California); Hsin-Hung Yang (University of Washington); and Mike Merges, Shawn Conley, and Kunio Nagashima (National Cancer Institute-Frederick Cancer Research and Development Center). We thank the following for intellectual input: Michael Piatak, Jr. and J. D. Lifson (Genelabs Technologies, Redwood City, CA), John Mills (Macfarlane Burnet Centre for Medical Research, Fairfield, Australia), John Sninsky (Roche Molecular Systems, Alameda, CA), Richard Tedder (University College of London Medical School, UK), and John Todd (Chiron).

References

1. Cohen J. Searching for markers on the AIDS trail. Science 1992;258:388–90.
2. Hammer S, Crumpacker C, D'Aquila R, et al. Use of virologic assays for the detection of human immunodeficiency virus in clinical trials: recommendations of the AIDS Clinical Trials Group Virology Committee. J Clin Microbiol 1993;31:2557–64.
3. Aboulker JP, Swart AM. Preliminary analysis of the Concorde trial. Lancet 1993;341:889–90.
4. Saag MS, Emini E, Laskin OL, et al. A short-term clinical evaluation of L-697,661, a non-nucleoside inhibitor of HIV-1 reverse transcriptase. N Engl J Med 1993;329:1065–72.
5. Nishanian P, Huskins KR, Stehn S, Detels R, Fahey JL. A simple method for improved assay that demonstrates that HIV p24 antigen is present as immune complexes in most sera from HIV-infected individuals. J Infect Dis 1990;162:21–8.
6. Coombs RW, Collier AC, Allain JP, et al. Plasma viremia in human immunodeficiency virus infection. N Engl J Med 1989;321:1626–31.
7. Coombs RW, Henrard DR, Mehaffey WF, et al. Cell-free plasma human immunodeficiency virus type 1 titer assessed by culture and immunocapture-reverse transcription–polymerase chain reaction. J Clin Microbiol 1993;31:1980–6.
8. Urdea MS. Synthesis and characterization of branched DNA (bDNA) for the direct and quantitative detection of CMV, HBV, and HIV. Clin Chem 1993;39:725–6.
9. Holodniy M, Katzenstein DA, Sengupta S, et al. Detection and quantification of human immunodeficiency virus RNA in patient serum by use of the polymerase chain reaction. J Infect Dis 1991;163:862–6.
10. Piatak M, Saag MS, Yang LC, et al. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. Science 1993;259:1749–54.
11. Semple M, Loveday C, Weller I, Tedder R. Direct measurement of viremia in patients infected with HIV-1 and its relationship to disease progression and zidovudine therapy. J Med Virol 1991;35:38–45.
12. Holodniy M, Katzenstein DA, Israelski DM, Merigan TC. Reduction in plasma human immunodeficiency virus ribonucleic acid after dideoxynucleoside therapy as determined by the polymerase chain reaction. J Clin Invest 1991;88:1755–9.
13. Holodniy M, Katzenstein D, Winters M, et al. Measurement of HIV virus load and genotypic resistance by gene amplification in asymptomatic subjects treated with combination therapy. J Acquir Immune Defic Syndr 1993;6:366–9.
14. Aoki-Sei S, Yarchoan R, Kageyama S, et al. Plasma HIV-1–infected individuals assessed by polymerase chain reaction. AIDS Res Hum Retroviruses 1992;8:1263–70.
15. Collier AC, Coombs RW, Fischl MA, et al. Combination therapy with zidovudine and didanosine compared to zidovudine alone in human immunodeficiency virus type one infection. Ann Intern Med 1993;119:786–93.
16. Winters MA, Tan LB, Katzenstein DA, Merigan TC. Biological variation and quality control of plasma human immunodeficiency virus type 1 RNA quantitation by reverse transcriptase-polymerase chain reaction. J Clin Microbiol 1993;31:2960–6.
17. Lori F, di Marzo Veronese F, De Vico AL, Lusso P, Reitz MS Jr, Gallo RC. Viral DNA carried by human immunodeficiency type 1 virions. J Virol 1992;66:5067–74.
18. Trono D. Partial reverse transcripts in virions from human immunodeficiency and murine leukemia viruses. J Virol 1992;66:4893–900.
19. Layne SP, Merges MJ, Dembo M, et al. Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus. Virology 1992;189:695–714.
20. Hollinger FB, Bremer JW, Myers LE, Gold JWM, McQuay L, the NIH/NIAID/DAIDS/ACTG Virology Laboratories. Standardization of sensitive human immunodeficiency virus coculture procedures and establishment of a multicenter quality assurance program for the AIDS Clinical Trials Group. J Clin Microbiol 1992;30:1787–94.
21. Hollinger FB, ed. ACTG manual for HIV laboratories. Version 2.0. Bethesda, MD: Division of AIDS, National Institute of Allergy and Infectious Diseases, 1993.
22. Kageyama S, Yamada O, Mohammad S, et al. An improved method for the detection of HIV antigen in blood of carriers. J Virol Methods 1988;22:125–31.

23. Whetsell AJ, Drew JB, Milman G, et al. Comparison of three nonradioisotopic polymerase chain reaction-based methods for detection of human immunodeficiency virus type 1. J Clin Microbiol 1992;30:845–53.
24. Mulder J, McKinney N, Christopherson C, Sninsky J, Greenfield L, Kwok S. A rapid and simple PCR assay for quantitation of HIV-1 RNA in plasma: application to acute retroviral infection. J Clin Microbiol 1994;32:292–300.
25. Henrard D, Mehaffey WF, Allain JP. A sensitive viral capture assay for detection of plasma viremia in HIV-infected individuals. AIDS Res Hum Retroviruses 1992;8:47–52.
26. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
27. Folks TM, Powell D, Lightfoote M, et al. Biological and biochemical characterization of a cloned leu-3$^-$ cell surviving infection with the acquired immune deficiency syndrome retrovirus. J Exp Med 1986;164:280–90.
28. Schochetman G, Sninsky JJ. Direct detection of human immunodeficiency virus infection using the polymerase chain reaction. In: Schochetman G, George JR, eds. AIDS testing methodology and management issues. New York: Springer-Verlag, 1992:90–110.
29. Johnson RF, Pickett SC, Barker DL. Autoradiography using phosphor technology. Electrophoresis 1990;11:355–60.

Volume 170 Number 3 September 1994

The Journal of Infectious Diseases

AN OFFICIAL PUBLICATION OF THE INFECTIOUS DISEASES SOCIETY OF AMERICA