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Quantitation of Human Immunodeficiency Virus Type 1 DNA by Two PCR Procedures Coupled with Enzyme-Linked Oligosorbent Assay

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Two quantitative PCR methods with our nonisotopic enzyme-linked oligosorbent assay (ELOSA) in microtiter plate format were developed for quantitation of human immunodeficiency virus type 1 (HIV-1). Quantitative competitive PCR (QC-PCR) was based on the coamplification of the wild-type *nef* region with a mimic competitive *nef* gene template carrying mutations in the capture region. Correlation of wild-type HIV-1 *nef* DNA to mimic template copy number permitted quantitation of HIV-1 copy numbers in the range of 20 to 2,000 copies per μg of DNA. Internally controlled PCR (IC-PCR) was based on coamplification of the *nef* region and the *ras* gene as an internal endogenous standard. Correlation to known amounts of HIV-1 DNA permitted quantitation by IC-PCR of HIV-1 copy numbers in the range of 10 to 2,000 copies per μg of DNA. QC- and IC-PCR–ELOSA were performed on a panel of 53 seropositive patients and 12 seronegative controls. The methods showed similar coefficients of variation below 24%. Quantitations by QC- and IC-PCR–ELOSA were identical for 77% of patient samples. The copy level ranged between 443 ± 156 and 21,453 ± 13,511 copies per 10^5 CD4 cells for asymptomatic and AIDS patients, respectively. The simplicity and reliability of QC- and IC-PCR–ELOSA methods make them appropriate for routine laboratory use in the quantitation of viral and bacterial DNAs.

Human immunodeficiency virus type 1 (HIV-1) proviral copy number is currently receiving much support as a marker for the clinical status of HIV-infected patients, in monitoring disease progression (6, 14, 28, 31), as a surrogate marker for mother-to-infant transmission (25), and as a marker to assess the efficacy of antiviral treatments (1, 3). Most quantitative techniques for HIV-1 detection are generally based on PCR. The exponential amplification of small amounts of nucleic acids makes PCR powerful but also challenging as a quantitative method. Variations in nucleic acid preparation, thermal cycler performance, choice of polymerase, and amplification procedure can cause large differences in the final product yield. To address the challenges of quantitative PCR, the procedure has been critically examined, leading to an understanding of the critical parameters involved in quantitative amplification. Accepted parameters can be summarized as a series of choices: external versus internal standard, exogenous versus endogenous standard, competitive versus noncompetitive amplification, and exponential versus plateau amplification (5, 13, 29).

The first quantitative approach with PCR was semiquantitative and based on the amplification of sample in limiting dilutions (28). Another approach to quantitation has been based on amplification with known amounts of an external standard, such as a cell line carrying a defined wild-type gene copy number (10, 15). These two approaches, however, fail to control tube-to-tube variation. Thus, alternative strategies including an internal standard have been developed. In quantitative competitive PCR (QC-PCR), the gene of interest is coamplified with different concentrations of an added standard; generally four different PCRs are done. The internal standard is defined to be as closely related as possible to the target, with differentiation occurring in the detection method, e.g., because of difference in size, hybridization sequence, or changes in restriction pattern (23, 24, 27). When the sequences of the target and the control are very close, this situation approaches the "equivalency of replication efficiencies" as defined by Nedelman et al. (22). The process of coamplification is truly competitive (29); therefore, PCR is performed to the plateau. The yield of generated target product can be directly correlated to the internal standard. In an alternate approach, the gene of interest is coamplified either with an endogenous standard such as the cellular HLA-DQ-α gene (16, 19) or with a fixed amount of a DNA fragment or plasmid that carries a heterologous sequence flanked by sequences homologous to the amplification primers (2, 7). Internal standards act as controls for amplification efficiency. However, as the sequences and the amount of target and control are different, the equivalency of replication efficiencies (22) is rarely approached, and therefore, the amplification process must be stopped during the exponential phase. The yield of generated target product can be directly correlated to standard curves.

The detection method must also be taken into consideration as a part of the overall quantitative process. The standardization of routine procedures, such as microtiter plate-based DNA and RNA hybridization assays (12, 17, 20), has considerably simplified this step of the quantitation procedure.

The aim of the present study was to define and compare two quantitative PCR methods that use internal standards and that

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TABLE 1. Primers, capture and detection probes, and synthetic templates used in this study[a]

Target gene	Oligonucleotide function(s)	DNA sequence (5′ to 3′)	Name	Location (nt)
NEF	PCR cloning	CCCGACAGGCCCGAAGGAA	U1353	7948–7966
	PCR mutagenesis	GGGTTACAGTAACGGATCCAATCACACTACT	L2000	8393–8365
	PCR mutagenesis	CCGTTACTGTAACCCAAAGAATGAGACGAGC	U1999	8379–8409
	PCR cloning	GTCCCCAGCGGAAAGTCCC	L390	8998–8980
	PCR	AAGATGGGTGGCAAITGGTC	AN635	8339–8358
	PCR	CATTGGTCTTAAAGGTACCTG	AN1116	8558–8578
	Capture	GGATGGCCTICTITAAGGGAAAGAATG	CN763	8375–8401
	Capture mut	GGATCCGTTACTGTAACCCAAAGAATG	CNM2001	8375–8401
	Detection	GAGGAGGTIGGTTTTCCAGTCA	DN753	8531–8552
	ELOSA positive control	CATTCTTTCCCTTACAGCAGGCCATCCttTGACTGGAAAACCCACCTCCTC	SN1336	
RAS	PCR	TGGTTATAGATGGTGAAACCTG	AR732	20–41
	PCR	CTGTAGAGGTTAATATCCGCAAA	AR170	157–179
	Capture	CAGTGCCATGAGAGACCAAT	CR1092	81–100
	Detection	TTCCTCTGTGTATTTGCCAT	DR284	121–140
	ELOSA positive control	ATTGGTCTCTCATGGCACTGttATGGCAAATACACAGAGGAA	SR1335	

[a] HIV-1 nomenclature is according to HIVHXB2R (18); the *Ras* exon 2 position is with reference to the HUMRASN2 sequence (Gen-Bank). Mismatches between the wild-type sequence and mutated sequences in the PCR primers used for mutagenesis (PCR mutagenesis) and in the capture probe (Capture mut) of the pNEFmut mimic template are underlined. Synthetic templates used for ELOSA quality control consisted of juxtaposed reverse complementary regions of capture and detection probes separated by two thymidines (in lowercase letters). nt, nucleotide.

were linked to our nonisotopic enzyme-linked oligosorbent assay (ELOSA) (9, 20), a detection assay based on sandwich hybridization in a microtiter plate format. Quality control for the ELOSA was first established prior to defining PCR procedures. The first PCR approach we analyzed was QC-PCR, which we based on the coamplification of the HIV-1 *nef* gene with different amounts of a pNEFmut plasmid that contains the *nef* region but with mutations in the capture probe recognition region. The NEF wild-type (NEF) and the NEF mimic (NEFmut) amplification products were differentiated in ELOSA by different capture probes and a common detection probe. Ratios of the optical density (OD) of NEFmut (NEFmut OD) to NEF OD were plotted against the number of mimic copies. The deduced linear curve was characterized by the equation and the coefficient of correlation, R^2. The R^2 cutoff was defined to validate QC-PCR amplification and to permit quantitation of HIV-1 copy number by the equation. We called our second PCR approach internally controlled PCR (IC-PCR). We based this approach on the coamplification of the HIV-1 *nef* gene with an internal endogenous standard, the *ras* gene, as a positive control of amplification. Acceptable OD intervals for RAS and NEF amplification products were defined to validate IC-PCR amplification and to quantitate HIV-1 copy number with an external standard with known amounts of HIV-1 DNA. We compared the reproducibilities and efficiencies of QC- and IC-PCR–ELOSA. These methods are simple and efficient means to quantify HIV-1 DNA in clinical samples.

MATERIALS AND METHODS

Patient samples, cell lines, and DNA preparation. Blood samples from 53 seropositive patients (10 at CDC stage II, 11 at CDC stage III, 6 at CDC stage IVA, 11 at CDC stage IVC2, 10 at CDC stage IVC1, and 5 at CDC stage IVD) were collected at the Hôpital Edouard Herriot, Lyon, France, and the Centre Hospitalier Universitaire Charles Nicolle, Rouen, France, and blood samples from 12 seronegative patients were kindly provided by D. Rigal, Blood Transfusion Center, Lyon, France, and J. M. Besnier, Centre Hospitalier Universitaire Bretonneau, Tours, France. The number of total cells, $CD4^+$ cells, and $CD8^+$ cells was determined by flow cytometry. The ACH-2 cell line which has been shown to contain one HIV proviral DNA copy per cell (6) and the control H9 lymphoid cell line were used. HIV DNA preparations from 5-ml blood samples or 2.0×10^7 cells were obtained with an Applied Biosystems model 340A nucleic acid extractor. DNA was quantified by spectrophotometry at 260 nm and analyzed on 0.8% agarose gels.

Oligonucleotide primers, probes, and synthetic templates. Oligonucleotides (Table 1) were prepared by the phosphoramidite method on an Applied Biosystems model 394 synthesizer and purified by reverse-phase high-performance liquid chromatography. Oligonucleotides used for capture and detection in ELOSA were synthesized with an amine arm at the 5′ end. This addition was performed on the synthesizer with the Aminolink II reagent (Applied Biosystems). Horseradish peroxidase labeling of detection oligonucleotides was performed as described by M. S. Urdea et al. (30). Primers and probes were chosen empirically and checked with OLIGO 4.03 software (National Bioscience, Plymouth, Minn.). The mutated NEF capture probe (Table 1) was designed by changing residues in the central core of the NEF wild-type capture probe by swapping G and C tracks: the length and T_m of both probes remained identical.

Internal standard for QC-PCR–ELOSA: plasmid pNEFmut construct. The HIV-HXB2R region from nucleotides 7948 to 8998 was PCR amplified with primers U1353 and L390 (Table 1) and cloned in plasmid PCRII (InvitroGen); this plasmid was named pNEF. The pNEFmut mimic template used in coamplification was synthesized by PCR-based site-specific mutagenesis, changing the NEF fragment in the capture probe region. PCRs involving overlapping mutagenesis primers (L2000 and U1999; Table 1) led to the generation of the left-side and right-side NEFmut fragments: primer pairs used were U1353-L2000 and U1999-L390, respectively. Five microliters of each reaction mixture was mixed and amplified with the external primers U1353 and L390, generating the full length NEFmut fragment. This product was cloned in plasmid PCRII and sequenced; this plasmid was named pNEFmut. The NEFmut sequence is equal in size and composition to the *nef* wild-type gene, except for the nine point mutations in the capture region.

QC-PCR. Four reactions were performed. Each reaction mixture contained 1 μg of DNA, 30 pmol of each NEF primer, 0, 20, 200, or 2,000 copies of linearized pNEFmut template, 250 μM deoxynucleoside triphosphates (dNTPs), and 1.5 U of *Taq* polymerase in 100 μl of PCR buffer consisting of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 0.01% gelatin. PCR was performed in a Perkin Elmer model 480A apparatus. Cycling conditions were 5 min at 80°C, and then 1.5 mM $MgCl_2$ was added and 45 cycles of standard PCR were performed. Each cycle consisted of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. The 45th cycle included a 7-min elongation step at 72°C.

External standard for IC-PCR–ELOSA. Two standard curves of HIV-1 DNA were established, with whole blood or cultured cells as a source of exogenous DNA. Four independent IC-PCRs were performed with known amounts of HIV-1 DNA extracted from ACH-2 cells mixed with DNA extracted from whole blood obtained from a pool of three HIV-negative volunteers. Ten independent IC-PCRs were performed with DNA extracted from 10 to 10^4 ACH-2 cells combined with 2.0×10^7 HIV-negative H9 cells. One microgram of DNA was IC-PCR amplified, and RAS and NEF amplification products were detected by ELOSA. NEF OD was plotted against HIV-1 copy number.

IC-PCR. Each reaction tube contained 1 μg of DNA, 30 pmol of each NEF primer, 5 pmol (or the concentration specified) of each RAS primer, 1.5 mM $MgCl_2$, 250 μM deoxynucleoside triphosphates, and 1.5 U of *Taq* polymerase (Perkin Elmer Cetus) in 100 μl of PCR buffer. Cycling conditions were 2 min at 95°C followed by 35 cycles of standard PCR. Each cycle consisted of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. The 35th cycle included a 7-min elongation step at 72°C.

TABLE 2. ELOSA quality control with synthetic templates[a]

Synthetic template (name)	Concentration (pM)	No. of tested plates	Mean OD	CV (%)
NEF (SN1336)	7.3	202	107	18
	21.8	198	325	17
	65.5	200	932	18
	196.5	69 + 128[b]	>2,142	IR[c]
RAS (SR1335)	57.5	70	486	18
	115	69	948	16

[a] NEF and RAS synthetic templates at specified concentrations were detected by ELOSA. ODs at 492 nm are expressed as ODs × 1,000. ODs are expressed as the means of independent ELOSA detections corresponding to the specified number of tested plates. CVs for each concentration of template are expressed.
[b] Number of plates with an OD greater than 2,500.
[c] IR, irrelevant.

ELOSA procedure. The ELOSA detection procedure (20) can be summarized in the following four steps: (i) denaturation of the PCR product and hybridization to capture probe preadsorbed to the microtiter plate, (ii) hybridization of the captured PCR product to the detection probe, (iii) washing and removing excess of detection probe, and (iv) colorimetric detection by addition of *O*-phenylenediamine substrate and reading the A_{492}. For quantitative ELOSA, 20 μl of the 100-μl amplification reaction mixture was diluted in 70 μl of hybridization buffer (0.1 M sodium phosphate, pH 7.0, 0.5 M NaCl, 0.65% Tween 20 [wt/vol], 0.14 mg of salmon sperm DNA [Boehringer] per ml, 2% polyethylene glycol 4000) and denatured by the addition of NaOH (final concentration, 0.2 M). After 5 min at room temperature, the sample was neutralized by the addition of acetic acid (final concentration, 0.2 M). The prepared DNA reaction mixture was adjusted, with the hybridization buffer, to 750 μl for IC-PCR or 2,000 μl for QC-PCR. A 50-μl aliquot of this solution was added to the well of a microtiter plate precoated with the RAS-, NEF-, or NEFmut-specific capture probe. Each sample was tested in duplicate. Hybridization to the capture and detection probes, washes, substrate addition, and signal reading were performed as previously described (20). ODs were expressed as A_{492} × 1,000.

ELOSA quality control. To control the quality of ELOSA reagents, 14 lots of solutions of 7.3, 21.8, 65.5, and 196.5 pM NEF synthetic template and 5 lots of solutions of 57.5 and 115 pM RAS synthetic templates (SN1336 and SR1335, respectively; Table 1) were prepared in hybridization buffer. Prepared lots were stable for 3 months at −20°C. Aliquots (50 μl) of each preparation were treated by ELOSA, but the denaturation step was omitted for these single-stranded templates.

Reproducibility of the PCR-ELOSA methods. Two DNA sources were tested, one consisting of DNA extracted from the whole blood of a seropositive patient and the other consisting of DNA extracted from 1,200 ACH-2 cells combined with 2.0×10^7 HIV-negative H9 cells. Ten independent IC-PCR and 10 independent QC-PCR amplifications were performed with HIV-1 DNA extracted from the seropositive patient sample, and 12 independent IC-PCR and 9 independent QC-PCR amplifications were performed with HIV-1 DNA extracted from mixed ACH-2 and H9 cells. PCR-ELOSA procedures were as described above, except that 0, 5, 50, and 500 copies of pNEFmut were used in QC-PCR.

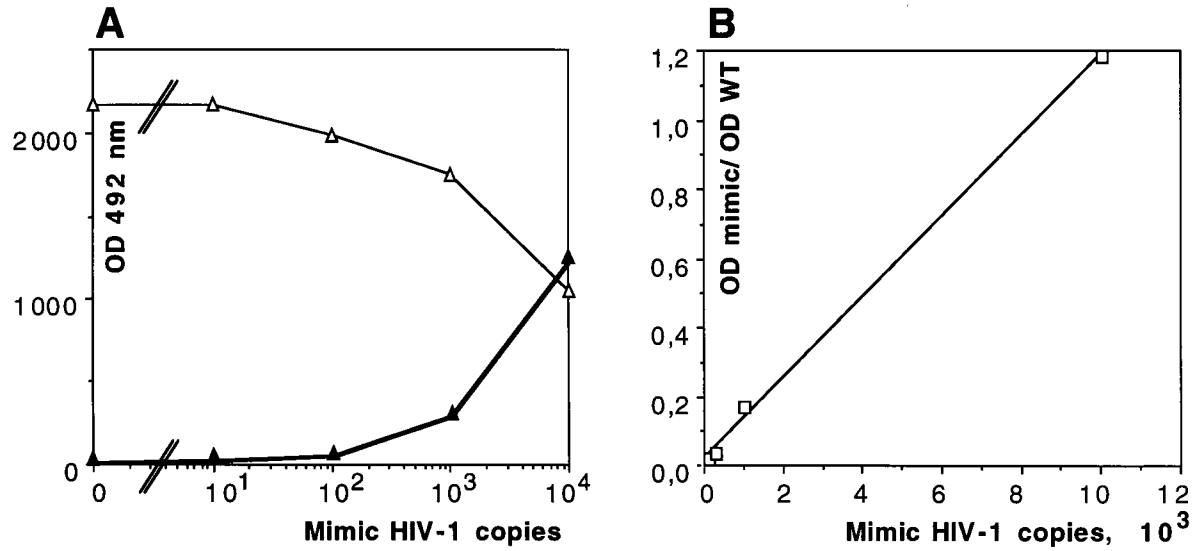
Statistical analyses. Patient sample populations were classified according to serological status (seronegative versus seropositive), $CD4^+$ lymphocyte levels (low, <200/μl; medium, 200 to 499/μl; high, ≥500/μl), or CDC stages (II and III, IVA and IVC2, and IVC1 and IVD). For IC-PCR–ELOSA, differences in the level of detection of the control cellular *ras* gene were analyzed by the Mann-Whitney U test (StatView II; Abacus Concepts, Inc., Berkeley, Calif.) for the serological groups and by the Kruskal-Wallis test (StatView II) for the $CD4^+$ lymphocyte level groups and CDC stage groups. The distribution of amplification acceptance criteria, R^2 for QC-PCR–ELOSA, and the RAS and NEF ODs for IC-PCR–ELOSA, were plotted by the box plot method (StatView II; Abacus Concepts, Inc.). HIV-1 copy numbers obtained by QC-PCR–ELOSA (QC_i) and IC-PCR–ELOSA (IC_i) were compared by the method of ratios (26); this method represents the quotient (q_i) as a function of QC_i ($q_i = IC_i/QC_i$) and shows the distribution of values and the margin of variation. Upper and lower limits for q_i were defined at 95 and 99%. Thresholds were determined by the formula $q = K \pm (K^2-1)^{1/2}$, where $K = 1/(1-t^2CV^2)$, t is the Student value, and CV is the coefficient of variation of the QC-PCR–ELOSA method.

RESULTS

ELOSA reagent quality control. Table 2 shows a linear correlation between NEF-SN1336 or RAS-SR1335 synthetic template concentrations and absorbance. NEF ODs ranged from 53 to 2,500 and RAS ODs ranged from 355 to 1,159. However, 65% of the reactions were saturated at the higher NEF-SN1336 concentration. CVs for the NEF and RAS systems ranged from 16 to 18%, independent of the synthetic template concentration.

QC-PCR–ELOSA. Control experiments showed that the nine changed residues in the NEFmut capture probe gave high specificity for pNEFmut PCR products (data not shown). The equivalency of replication efficiency was then assessed by mixing known amounts of the circular plasmids pNEF and pNEFmut. QC-PCR was performed for 45 cycles as described in Materials and Methods, except that the pNEFmut plasmid was circular and $MgCl_2$ was added at the beginning of the reaction. pNEF and pNEFmut QC-PCR amplification products were distinguished by ELOSA. Figure 1A shows a typical curve of pNEF amplification inhibition by increasing pNEFmut concentration. Plots were simplified by expressing the ratio of mimic and wild-type detection signals, which led to a linear curve showing OD ratios as a function of pNEFmut copy number (Fig. 1B). This was determined for 10, 100, 1,000, and 10,000 copies of HIV-1 pNEF. R^2 ranged from 0.999 to 1.000 (Fig. 1C). However, much variation between the expected and deduced copy number was observed at the low-copy-number level. As we wanted to quantify at least 10 copies of HIV-1 in 1 μg of DNA, the QC-PCR method was modified. We observed that the sensitivity of QC-PCR could be improved by (i) linearization of the pNEFmut plasmid and (ii) inclusion of a hot start followed by addition of $MgCl_2$ (data not shown). R^2 was used as the acceptance criterion to validate the QC-PCR–ELOSA experiments. When the experimental R^2 was greater than the accepted R^2 cutoff of 0.985, the HIV-1 copy number could be quantified with the plotted curve.

IC-PCR–ELOSA. The RAS primer concentrations that permit efficient amplification of the *ras* gene without affecting amplification of the *nef* gene were determined. DNA was extracted from mixtures of HIV-infected ACH-2 cells and noninfected H9 cells, and *nef* and *ras* genes were coamplified with 30 pmol of NEF primers and 0, 2.5, 5, 10, and 20 pmol of RAS primers as described in Materials and Methods. RAS and NEF IC-PCR amplification products were detected by ELOSA. The lowest RAS primer concentration, 2.5 pmol/100-μl PCR mixture, did not permit significant amplification of *ras*, whereas the highest primer concentration, 20 pmol/100-μl PCR mixture, inhibited *nef* amplification (data not shown). It was determined that efficient amplification of the *ras* gene which did not affect amplification of the *nef* gene was achieved at 5 and 10 pmol of RAS primers in coamplification (Fig. 2A). The plots of log (NEF OD) as a linear function of log (HIV-1 copy number) with 5 pmol of RAS primers, however, more closely approached the amplification of *nef* in the absence of RAS primers than did that with 10 pmol of RAS primers (Δintercept = 6% with Δslope = 3% for 5 pmol versus Δintercept = 64% with Δslope = 12% for 10 pmol). Only slight reductions of the RAS (from 7 to 23%) and NEF (from 0 to 24%) signals were generated in coamplification compared with that generated in single amplification, irrespective of the amount of HIV-1 (Fig. 2A). Then, standard curves were determined with 30 pmol of each NEF primer and 5 pmol of each RAS primer. RAS ODs ranged from 197 to 933 and 254 to 1,742 in the presence of exogenous DNA from whole blood and H9 cells, respectively. Plots of OD readings against HIV-1 copy number were different depending on the exogenous DNA source, whole blood (Fig. 2B) or H9 cells (data not shown). Curves extrapolated as log (OD) = a[log (HIV-1 copy number)] + b permitted an accurate determination of HIV copy number (Fig. 2C). Acceptance criteria to validate IC-PCR–ELOSA experiments were defined by OD intervals for RAS and NEF. As RAS ODs



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Expected copy number	R^2	Regression curves: OD mimic /OD WT = a (copies) + b		Measured copy number
		a: slope	b: intercept	
10	1.000	125.58	-7.1955	65
100	0.999	3.2581	+0.3477	200
1,000	0.999	0.7242	+0.2210	1078
10,000	0.999	0.1168	+0.0233	8362

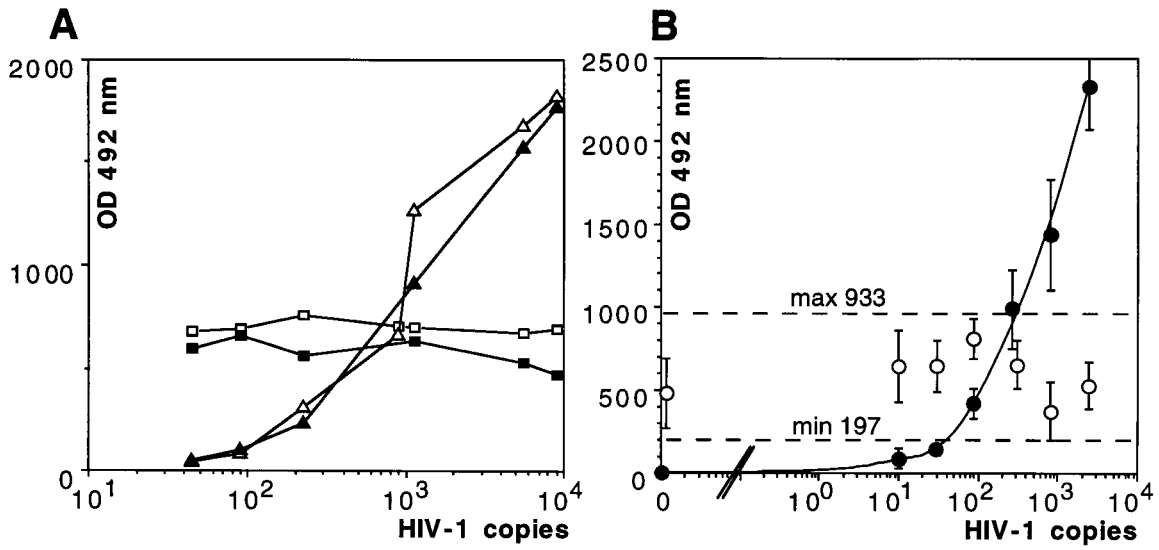
FIG. 1. Definition of QC-PCR–ELOSA procedure. (A) NEF (△) and NEFmut (▲) ODs determined by QC-PCR–ELOSA in relation to pNEFmut (mimic) plasmid copy number. QC-PCR was performed to the plateau, with 10,000 copies of pNEF as wild-type template and 10 to 10,000 copies of pNEFmut as internal standard, and PCR products were detected by ELOSA as described in Materials and Methods. ODs at 492 nm are expressed as ODs × 1,000. (B) NEF OD/NEFmut OD (OD mimic/OD WT) ratio in relation to mimic plasmid copy number. The QC-PCR–ELOSA experiment was the same one described for panel A. (C) QC-PCR–ELOSA quantitation of 10 to 10,000 copies of pNEF plasmid. The measured copy numbers were calculated with linear regression curves of the OD mimic/OD WT ratio in relation to mimic plasmid copy number. The regression curves were characterized by coefficient of correlation (R^2), slope, and intercept. Values for slopes are reported as $\times 10^3$.

differed between whole blood and H9 cells, the RAS acceptance criterion was fixed at an OD between 200 and 1,500. The NEF acceptance criterion was fixed at an OD between 90 and 2,300 for whole blood samples and between 100 and 1,900 for cultured cells samples. When the experimental RAS and NEF ODs fit these criteria, HIV-1 copy number could be determined by using the equation of the relevant standard curve (Fig. 2C).

Reproducibility of the PCR-ELOSA methods. Table 3 shows the means and CVs of deduced HIV-1 copy number and R^2 ranges for QC-PCR–ELOSA and NEF and RAS OD ranges for IC-PCR–ELOSA. The data fit acceptance criteria of both methods. CVs of the deduced copy numbers were below 24 and 20% for QC-PCR–ELOSA and IC-PCR–ELOSA, respectively.

Sample analysis by QC- and IC-PCR–ELOSA methods. The sample panel consisted of 53 seropositive patients. Of the 53 samples, 51 fit the acceptance criterion for QC-PCR–ELOSA; two seropositive patients had R^2s of <0.985 (Fig. 3). Of the 53 seropositive samples, 49 fit the acceptance criterion for IC-PCR–ELOSA; one asymptomatic patient exhibited a RAS OD of <200 and three seropositive patients had NEF ODs of <90 (Fig. 3). No significant difference in the detection level of the control cellular gene between 12 seronegative controls (data not shown) and the 53 seropositive patients was observed (P = 0.1239 [Mann-Whitney U test]).

To relate the number of proviral copies to 10^5 CD4 cells, the proviral copy number in 1 μg of DNA, which corresponded to 150,000 cells, was multiplied by the correction factor (0.666) and divided by the ratio of CD4 cells to total lymphocytes. Proviral copy numbers were determined for the 48 patients fitting the acceptance criteria of both methods. Limiting dilutions were also used in combination with PCR and ELOSA (LD-PCR–ELOSA) to quantify HIV copy numbers (data not shown). On the basis of the NEF OD cutoff previously defined (20), HIV copy numbers were separated into four classes: less than 50, 50 to 500, 500 to 5,000, and 5,000 to 50,000 HIV copies per 10^5 $CD4^+$ cells. Under this classification, copy numbers determined by the different assays showed the following correlations: QC-, IC-, and LD-PCR–ELOSA, 35%; LD- and QC-PCR–ELOSA, 42%; LD- and IC-PCR–ELOSA, 40%; and IC- and QC-PCR–ELOSA, 83%. By using absolute copy numbers instead, QC- and IC-PCR–ELOSA could be compared by the ratio method (Fig. 4). Ratios were not different from 1 for 37 of 48 samples (77%) within the 95% confidence interval,



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Source of exogenous DNA	RAS OD mean	RAS OD sd	NEF OD min	NEF OD max	Regression curves: Log (OD) = a Log (copies) + b R^2	a: slope (CV%)	b: intercept (CV%)
whole blood	593	197	90	2333	0.9742	0.6242 (8%)	1.3417 (9%)
H9 cells	939	297	97	1964	0.9944	0.6365 (5%)	1.4290 (5%)

FIG. 2. Definition of IC-PCR–ELOSA procedure. (A) Definition of NEF and RAS primer concentrations in IC-PCR. NEF and RAS ODs in relation to HIV-1 copy number and simultaneous presence or absence of NEF and RAS primers were determined by PCR-ELOSA. DNAs (1 μg) were PCR amplified with either 30 pmol of NEF primers or 5 pmol of RAS primers and NEF (△) and RAS (□) PCR products were detected by ELOSA; DNAs were PCR coamplified with 30 pmol of NEF primers and 5 pmol of RAS primers, and NEF (▲) and RAS (■) PCR products were detected by ELOSA as described in Materials and Methods. (B) Definition of standard curves in IC-PCR–ELOSA. NEF and RAS ODs in relation to HIV-1 copy number were determined by IC-PCR–ELOSA. Four independent amplifications were performed with 1 μg of DNA, consisting of known amounts of DNA extracted from ACH-2 cells combined with DNA extracted from total blood obtained from a pool of three HIV-negative, healthy volunteers. NEF (●) and RAS (○) PCR products were detected by ELOSA. Bars show standard deviations. (C) Characteristics of the standard curves with whole blood (in panel B) or H9 cells as exogenous DNA. Characteristics shown include RAS OD means and standard deviations (sd), NEF OD minima (min) and maxima (max) defining the range of validity of each regression curve, coefficients of correlation (R^2), and slope and intercept with their respective coefficients of variation (CV%).

and ratios for all samples (100%) fell within the 99% confidence interval (Fig. 4).

When the values which fell within the 95% confidence level were retained, HIV copy number was shown to range from 115 to 56,467 per 10^5 CD4 cells. A highly significant difference in the quantitation of HIV provirus was observed for patients classified either by CDC stage ($P = 0.0028$ and $P = 0.0068$ for QC- and IC-PCR–ELOSA, respectively [Kruskal-Wallis test]) or by CD4 level ($P = 0.0001$ for both QC- and IC-PCR–ELOSA [Kruskal-Wallis test]). Variations in copy number were observed in each classification group (Table 4); e.g., HIV copy numbers in the asymptomatic group defined by CDC stages II and III ranged from 245 to 12,409 per 10^5 CD4 cells. We observed an 8-fold increase in provirus from CDC stages II and III to IVC1 and IVD, a 32-fold increase in provirus that accompanied CD4 cell decrease, and a 48-

TABLE 3. Reproducibility of IC- and QC-PCR–ELOSA[a]

Sample source	QC-PCR–ELOSA data				IC-PCR–ELOSA data					
	R^2		NEF copies		RAS OD		NEF OD		NEF copies	
	Min	Max	Mean	CV (%)	Min	Max	Min	Max	Mean	CV (%)
Patient	0.988	0.999	196	24	302	555	598	777	264	11
Cell line	0.998	1.000	154	15	356	699	464	712	136	20

[a] QC- and IC-PCR–ELOSA procedures were performed on two different DNA samples as described in Materials and Methods. Minima (Min) and maxima (Max) of the acceptance criteria, R^2 for QC-PCR–ELOSA and RAS and NEF ODs for IC-PCR–ELOSA, are indicated. Means and CVs of the deduced copy numbers are also indicated.

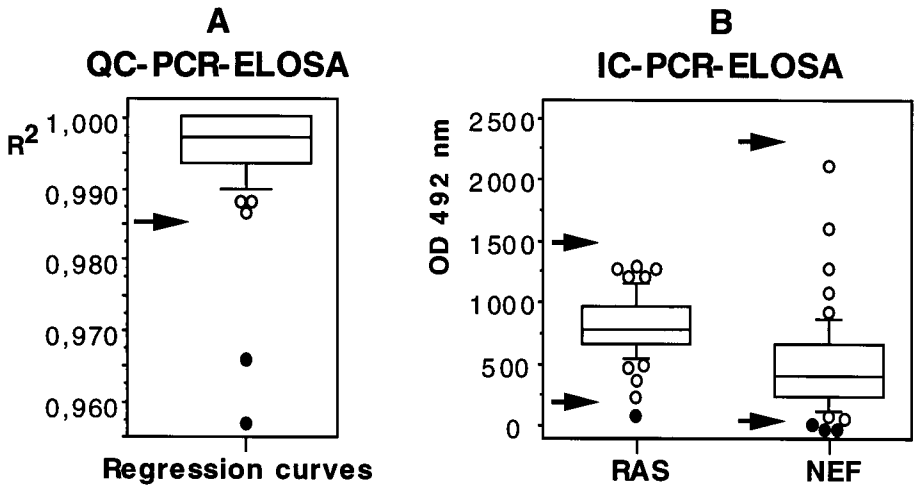

FIG. 3. Distribution of the acceptance criteria determined for PCR-ELOSA methods. Box plots indicate the distribution, the median OD, and the 10th, 25th, 50th, 75th, and 90th percentiles. Data fitting (○) or not fitting (●) acceptance criteria are plotted. (A) Distribution of the coefficients of correlation, defined as the NEF OD/NEFmut OD ratio in relation to pNEFmut mimic plasmid copy number, of the regression curves for QC-PCR–ELOSA is shown. The R^2 cutoff of 0.985 is shown by an arrow. (B) Distribution of the ODs determined by IC-PCR–ELOSA, in relation to RAS and NEF oligonucleotide quartets. Acceptance criteria (200 < RAS OD < 1,500 and 90 < NEF OD < 2,300) are shown by arrows.

fold increase in copy number between the group defined by CDC stages II and III with high CD4 cell count and the group defined by CDC stages IVC1 and IVD with low CD4 cell count (Table 4).

DISCUSSION

The aim of this study was to define and compare two quantitative PCR methods linked to our nonisotopic ELOSA detection assay (20). We used the previously described NEF system (20) because it (i) was shown to detect at least 10 copies of HIV-1 DNA in a background of 1 μg of DNA, (ii) was 100% specific and 95% sensitive for a 63-patient panel, and (iii) gave OD readings significantly greater than background.

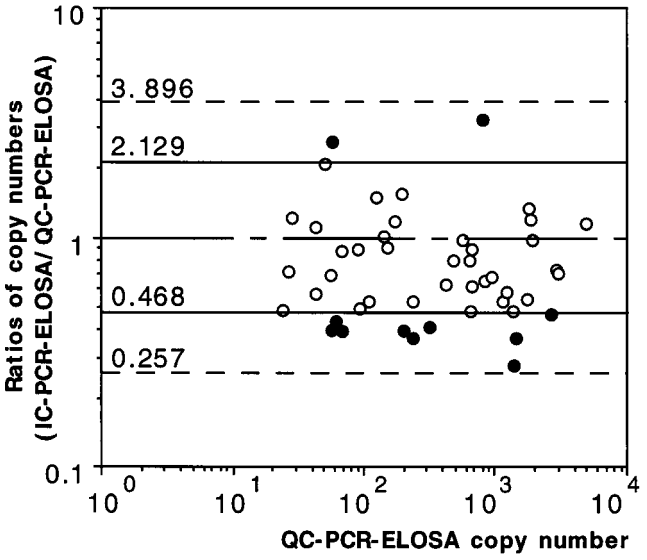

FIG. 4. Comparison of QC- and IC-PCR–ELOSA by the method of ratios. A total of 48 HIV-1 DNA samples were quantified by QC- and IC-PCR–ELOSA. The ratio of IC-PCR–ELOSA copy number to QC-PCR–ELOSA copy number was drawn as a function of QC-PCR–ELOSA copy number. The optimal ratio of 1 is indicated (—— – ——) as well as 95% (——) and 99% (– – –) upper and lower limits of confidence intervals deduced with a 24% coefficient of variation for QC-PCR–ELOSA. ○, data within the 95% confidence interval; ●, data between the 95 and 99% confidence interval.

Synthetic NEF and RAS templates provided good quality control systems for ELOSA. The lowest NEF-SN1336 concentration (7.3 pM) gave a reading 12 times greater than the previously described cutoff (20), and the higher NEF-SN1336 concentration (196.5 pM) gave a reading near saturation in the automatic microtiter plate assay. The RAS synthetic templates gave readings that fell within the detection levels observed after amplification of 1 μg of sample DNA. However, the CV of 18% was significantly higher than that previously observed with ELOSA (7% [20]). This might be explained by the increased number of independent preparations of target sequence (14 versus 2) and by the increased number of analyzed plates (200 versus 10). This discrepancy indicates the difficulty in obtaining reproducible batches of synthetic template at low concentrations.

The main difficulty with using internal standards is the competition resulting from coamplification of the gene of interest and a sequence of reference (29). Multiple sets of primers in the same reaction generally interfere with amplification of either the target sequence and/or the reference sequence. Furthermore, differences in the composition and quantity of the gene of interest and of the reference sequence may influence amplification efficiency. To address these problems, we defined QC-PCR–ELOSA as a true competitive quantitative process and IC-PCR–ELOSA as a positive control-based quantitative process.

Quantitation by QC-PCR–ELOSA was determined with plots of ratios of NEFmut OD to NEF OD against the number of mimic copies. The linear curve produced permitted quantitation when the coefficient of correlation was greater than the R^2 cutoff. Curves obtained by QC-PCR–ELOSA were similar to those previously obtained by ethidium bromide fluorescence and computer video imaging (27), radiolabeled PCR products and scintillation counting (27), dye-labeled oligonucleotide fluorescence and an automated sequencer (23), or digoxigenin-labeled probe and a microtiter plate assay (17).

Quantitation by IC-PCR–ELOSA was determined with a defined OD range for RAS, and subsequent quantitation was

TABLE 4. Distribution of HIV-1 provirus copy numbers in relation to CDC stages and $CD4^+$ cell levels[a]

No. of $CD4^+$ cells/μl	Copy no. (mean ± SD) at CDC stage			Copy no. (mean ± SD) for $CD4^+$ level
	II–III	IVCA–IVC2	IVC1–IVD	
≥500	443 ± 156			443 ± 156
200 to 499	1,324 ± 1,087	2,596 ± 3,047	3,439 ± 3,464	2,030 ± 2,322
<200	7,041 ± 2,866	8,800 ± 7,868	21,453 ± 13,511	13,971 ± 12,070
Total	2,232 ± 2,856	6,584 ± 7,158	18,177 ± 14,144	

[a] CDC stage groups were joined as three groups, II and III (asymptomatic), IVA and IVC2 (AIDS-related complex), and IVC1 and IVD (AIDS), and combined with the three levels of CD4+ cells shown, describing essentially the new A-B-C/1-2-3 CDC classification (4). The final row and column show the provirus distributions according to CDC stage and $CD4^+$ level, respectively. The mean copy numbers and standard deviations per class are indicated.

made by reference to external standard curves within a defined NEF OD range. The determined ratio of NEF and RAS primers used in IC-PCR (30 pmol/5 pmol) was valid only within a short range of DNA concentration (data not shown). Thus, the RAS endogenous standard cannot be used as an absolute quantitative marker of DNA input, as suggested by others (16, 19) and previously hypothesized by us (20). As no difference in the detection of the control cellular gene between seronegative and seropositive samples was observed, RAS conditions for coamplification were considered suitable for use as a positive control of DNA integrity and amplification efficiency (1, 7, 8). Standard curves of log (OD) as a linear function of log (HIV-1 copy number) were used to determine HIV-1 copy number. This representation was accurate within the ranges of 10 to 1,762 copies of HIV-1 in 1 μg of DNA from whole blood and 7 to 848 copies of HIV-1 in 1 μg of DNA from H9 cells. The sensitivity we observed and the range of quantitation correlate well with results described by others: 1 to 1,000 copies (19), 12 to 400 copies (16), 10 to 1,000 copies (31), and 100 to 10,000 copies (1).

The reproducibilities of QC- and IC-PCR–ELOSA varied, with CVs from 11 to 20% and 15 to 24%, respectively. These CVs are comparable to those described by others: from 2.2 to 39.9% (16) and 9.6% (1) for IC-PCR-like formats linked to isotopic detection and from 8.2 to 28.9% (29) and 25% (23) for QC-PCR-like formats linked to nonisotopic detection. Variation in the PCR-ELOSA protocols was determined to be less than 24%, which is relatively efficient compared with the CV of 18% obtained with synthetic templates used in the ELOSA quality control assay (see above). As discussed by Ferre (13), the established limits of a quantitative PCR method are reliable only when the same batch of standard is used within a given study. For QC-PCR–ELOSA, the difference between the expected and determined copy number, at the 10-copy level, may have reflected the difficulty in obtaining reproducible batches of standard at low concentrations. Furthermore, the context of the standard appeared critical as linearized plasmid was more efficient than circular plasmid. With IC-PCR–ELOSA, RAS OD ranges and standard curves for whole blood and cell line samples differed. This difference could be either a result of the nature of the samples or due to sample variation. Therefore, standard curves should be defined according to the source of the sample, the nature of the sample, and sample treatment.

QC- and IC-PCR–ELOSA were tested on a sample panel consisting of 53 seropositive patients at different CDC stages and 12 seronegative controls. Quantitation was possible within acceptance criteria for 96% of samples for QC-PCR–ELOSA and for 92% of samples for IC-PCR–ELOSA. In contrast, the LD-PCR–ELOSA correlated poorly with these two methods. The discrepancy between LD-PCR–ELOSA and internal standard-based methods may have been attributable to the absence of a control for tube-to-tube variation in the former. It may also have reflected that the NEF OD cutoff used in LD-PCR–ELOSA was not suitable, as linear relationship between the initial amount of target and the amplification product was only maintained for a limited range of starting DNA, as previously described (11). Quantitations by QC- and IC-PCR–ELOSA were identical for 77% of patient samples, with a 95% confidence level, on the basis of the ratio method. Discrepancies were not the result of a difference in the sensitivities of these methods, since discrepancies were not localized at low-copy-number levels but spread throughout the concentration range of 600 to 3,000 copies. Discrepancies between these methods were also not due to the chosen acceptance criteria, as R^2 and RAS and NEF ODs were distributed randomly within their respective acceptability ranges. On the other hand, discrepancy could be due to the method of analysis: an underestimated CV in QC-PCR–ELOSA could result in a too stringent test, which was possible since CV was defined with only one target concentration.

HIV copy level among individuals ranged from 115 to 56,467 copies per 10^5 CD4 cells. This is similar to the previously observed ranges of 1 to 10,000 copies per 10^5 CD4 cells (31); high levels of 40,000 to 50,000 HIV-1 copies per 10^5 CD4 cells have also been observed (10, 31). We observed, as have others, an increase in proviral copies with progression to symptomatic infection and with a decrease in $CD4^+$ cell count (14, 28, 31). HIV copy level showed a 48-fold increase from asymptomatic to AIDS stages. On the other hand, viral burden, defined as provirus copy number per milliliter of blood, showed only a fourfold increase, which correlates with the threefold increase described by Yerly et al. (31). These observations suggest that, in peripheral blood, an increased number of infected cells is counterbalanced by a decrease in cells susceptible to infection during the progression of the illness.

In conclusion, we have described two quantitative PCR procedures coupled to our ELOSA microtiter plate assay. For quantifying HIV DNA, IC-PCR–ELOSA seemed to be the method of choice. The method requires only one tube and includes a single control for DNA integrity and amplification efficiency. On the other hand, for quantifying HIV viral RNA, QC-PCR–ELOSA seems to be the method of choice since exogenous nucleic acids controls are not present. This was recently demonstrated by continuous reverse transcription PCR (21) when in vitro-synthesized RNA NEFmut was added directly to the reaction tube (19a). Although PCR-ELOSA-based quantitative methods are simple and reliable, improvements are necessary to define routine diagnostic tests wherein each step of the process is controlled. The two main points to resolve are the pre-PCR step, consisting of sample preparation and calibration, and the quantitative PCR step, which is de-

pendent on obtaining reliable batches of internal and external standards.

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REFERENCES

1. **Aoki, S. A., R. Yarchoan, R. V. Thomas, J. M. Pluda, K. Marczyk, S. Broder, and H. Mitsuya.** 1990. Quantitative analysis of HIV-1 proviral DNA in pheripheral blood mononuclear cells from patients with AIDS or ARC: decrease of proviral DNA content following treatment with 2′,3′-dideoxyinosine (ddl). AIDS Res. Hum. Retroviruses **6:**1331–1339.
2. **Arnold, B. L., K. Itakura, and J. J. Rossi.** 1992. PCR-based quantitation of low levels of HIV-1 DNA by using an external standard. Genet. Anal. Tech. Appl. **9:**113–116.
3. **Bruisten, S. M., M. H. G. M. Koppelman, M. T. L. Roos, A. E. Loeliger, P. Reiss, C. A. B. Boucher, and H. G. Huisman.** 1993. Use of competitive polymerase chain reaction to determine HIV-1 levels in response to antiviral treatments. AIDS **7**(Suppl. 2)**:**S15–S20.
4. **Centers for Disease Control and Prevention.** 1992. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. Morbid. Mortal. Weekly Rep. **41**(No. RR-17)**:**1–20.
5. **Clementi, M., S. Menzo, P. Bagnarelli, A. Manzin, A. Valenza, and P. E. Varaldo.** 1993. Quantitative PCR and RT-PCR in virology. PCR Methods Applic. **2:**191–196.
6. **Clouse, K. A., D. Powell, I. Washington, G. Poli, K. Strebel, W. Farrar, P. Barstad, J. Kovacs, A. S. Fauci, and T. M. Folks.** 1989. Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone. J. Immunol. **142:**431–438.
7. **Cone, R. W., A. C. Hobson, and M.-L. W. Huang.** 1992. Coamplified positive control detects inhibition of polymerase chain reactions. J. Clin. Microbiol. **30:**3185–3189.
8. **Coutlée, F., Y. He, P. Saint-Antoine, C. Olivier, and A. Kessous.** 1995. Coamplification of HIV type 1 and β-globin gene DNA sequences in a nonisotopic polymerase chain reaction assay to control for amplification efficiency. AIDS Res. Hum. Retroviruses **11:**363–371.
9. **Cros, P., P. Allibert, B. Mandrand, J.-M. Tiercy, and B. Mach.** 1992. Oligonucleotide genotyping of HLA polymorphism on microtitre plates. Lancet **340:**870–873.
10. **Dickover, R. E., R. M. Donovan, E. Goldstein, S. Dandekar, C. E. Bush, and J. R. Carlson.** 1990. Quantitation of human immunodeficiency virus DNA by using the polymerase chain reaction. J. Clin. Microbiol. **28:**2130–2133.
11. **Diviacco, S., P. Norio, L. Zentilin, S. Menzo, M. Clementi, G. Biamonti, S. Riva, A. Falaschi, and M. Giacca.** 1992. A novel procedure for quantitative polymerase chain reaction by co-amplification of competitive templates. Gene **122:**3013–3020.
12. **Dyster, L. M., L. Abbott, V. Bryz-Gornia, B. J. Poiesz, and L. D. Papsidero.** 1994. Microplate-based DNA hybridization assays for detection of human retroviral gene sequences. J. Clin. Microbiol. **32:**547–550.
13. **Ferre, F.** 1992. Quantitative or semi-quantitative PCR: reality versus myth. PCR Methods Applic. **2:**1–9.
14. **Genesca, J., R. Y. H. Wang, H. J. Alter, and J. W. K. Shih.** 1990. Clinical correlation and genetic polymorphism of the human immunodeficiency virus proviral DNA obtained after polymerase chain reaction amplification. J. Infect. Dis. **162:**1025–1030.
15. **Innocenti, P., M. Ottmann, P. Morand, P. Leclercq, and J. M. Seigneurin.** 1992. HIV-1 in blood monocytes: frequency of detection of proviral DNA using PCR and comparison with the total CD4 count. AIDS Res. Hum. Retroviruses **8:**261–268.
16. **Kellog, D. E., J. J. Sninsky, and S. Kwok.** 1990. Quantitation of HIV-1 proviral DNA relative to cellular DNA by polymerase chain reaction. Anal. Biochem. **189:**202–208.
17. **Kohsaka, H., A. Taniguchi, D. D. Richman, and D. A. Carson.** 1993. Microtiter format gene quantification by covalent capture of competitive PCR products: application to HIV-1 detection. Nucleic Acids Res. **21:**3469–3472.
18. **Lawrence, J., and K. Macinnes.** 1990. HIV and SIV nucleic acid sequences, p. I.A.68–I.A.71. *In* G. Myers, J. A. Berzofsky, A. B. Rabson, T. F. Smith, and F. Wong-Staal (ed.), Human retrovirus and AIDS 1990: a compilation and analysis of nucleic acid and amino acid sequences. Theoretical Biology and Biophysics Group T-10, Los Alamos National Laboratory, Los Alamos, N.M.
19. **Lee, T. H., F. J. Sunzeri, L. H. Tobler, B. G. Williams, and M. P. Busch.** 1991. Quantitative assessment of HIV-1 DNA load by co-amplification of HIV-1 gag and HLA-DQ-a genes. AIDS **5:**683–691.

19a. **Mallet, F.** Unpublished data.

20. **Mallet, F., C. Hebrard, D. Brand, E. Chapuis, P. Cros, P. Allibert, J.-M. Besnier, F. Barin, and B. Mandrand.** 1993. Enzyme-linked oligosorbent assay for detection of polymerase chain reaction-amplified human immunodeficiency virus type 1. J. Clin. Microbiol. **31:**1444–1449.
21. **Mallet, F., G. Oriol, C. Mary, B. Verrier, and B. Mandrand.** 1995. Continuous versus uncoupled reverse transcription-PCR procedure using AMV-RT and Taq polymerase. BioTechniques **18:**678–687.
22. **Nedelman, J., P. Heagerty, and C. Lawrence.** 1992. Quantitative PCR: procedures and precisions. Bull. Math. Biol. **54:**477–502.
23. **Pannetier, C., S. Delassus, S. Darche, C. Saucier, and P. Kourilsky.** 1993. Quantitative titration of nucleic acids by enzymatic amplification reactions run to saturation. Nucleic Acids Res. **21:**577–583.
24. **Piatak, M., Jr., K.-C. Luk, B. Williams, and J. D. Lifson.** 1993. Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species. BioTechniques **14:**70–81.
25. **Roques, P., D. Marce, C. Courpotin, F. Parnet Mathieu, F. Herve, F.-D. Boussin, R. Narwa, M.-C. Meyohas, C. Dollfus, and D. Dormont.** 1993. Correlation between HIV provirus burden and *in utero* transmission. AIDS **7**(Suppl. 2)**:**S39–S43.
26. **Sholler, R., G. Gervasi, R. Avigdor, and M. Castanier.** 1987. Comparison de deux méthodes de dosage, p. 225–233. *In* Colloque sur les actualités en immunoanalyse. CORATA, Université de Bordeaux II, Bergeret, Bordeaux, France.
27. **Siebert, P. D., and J. W. Larrick.** 1993. PCR MIMICS: competitive DNA fragments for use as internal standards in quantitative PCR. BioTechniques **14:**244–249.
28. **Simmonds, P., P. Balfe, J. F. Peutherer, C. A. Ludlam, J. O. Bishop, and A. J. Leigh Brown.** 1990. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. J. Virol. **64:**864–872.
29. **Sninsky, J. J., and S. Kwok.** 1993. The application of quantitative polymerase chain reaction to therapeutic monitoring. AIDS **7**(Suppl. 2)**:**S29–S34.
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. **Yerly, S., E. Chamot, B. Hirschel, and L. H. Perrin.** 1992. Quantitation of human immunodeficiency virus provirus and circulating virus: relationship with immunologic parameters. J. Infect. Dis. **166:**269–276.