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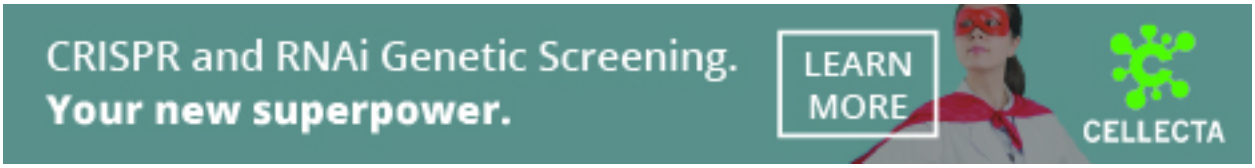

An Internally Controlled Virion PCR for the Measurement of HIV-1 RNA in Plasma

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We have developed an assay to measure the HIV-1 RNA in patients' plasma or sera using an infectious mutant virus as an internal control. The mutant virus VX-46 has a 25-bp insert in a conserved region between the primer-binding and major splice donor sites. To utilize this virus as an internal control, different dilutions of this virus were added to aliquots of plasma sample to be measured, RNA was isolated and reverse-transcribed to cDNA. PCR was performed with primers selected to include the sequences on either side of the insert contained in the externally added virus. The DNA product from the control virus is 25 bp longer than that from the virus present in plasma. The amount of viral RNA present in a plasma sample is calculated after the PCR-amplified products are separated by gel electrophoresis. Unlike other quantitative PCR assays, this internally controlled virion PCR (ICVPCR) assay eliminates errors introduced by variable recovery during the RNA purification step, therefore, enhancing the accuracy of the assay.

Quantitative PCR has been used to measure the relative levels of RNA and DNA from a variety of different samples. Under ideal conditions a direct correlation can be found between the amount of starting target material and the amount of PCR product.[1–3] However, this is often not the case for clinical samples owing to the presence of inhibitors of PCR in samples and differing efficiencies in sample recovery and kinetics of PCR.[4–7] Small variations in amplification efficiency can change the yield of the product and make it difficult to accurately estimate the amount present in the starting material.[8] To avoid these problems, several laboratories have described the use of internal standards in PCR.[8–14] Generally, the internal standard DNA or RNA share the same primers as the target DNA or RNA but will contain either a deletion or an insertion so that the products obtained from the standard and target can be distinguished.

A variation in the use of an internal control is the competitive PCR procedure.[8] In this method varying known amounts of the internal standards are added to equal aliquots of the sample containing the unknown target sequence. The internal standard and the target sequence compete equally for primer binding and amplification in the PCR. Variables such as the efficiency of amplification and the number of cycles will have the same effect on both templates. Equal amounts of products will be formed when the initial concentrations of the templates are equal. Experimentally, the ratio of products formed can be determined and the equivalence point can be calculated. This method has been successfully employed to quantify the amount of HIV-1 RNAs in clinical samples.[15,16] However, this method does not control the variations in RNA recovery from sample to sample. Here, we report a modified method in which an infectious HIV-1 mutant virus was used as the source of competitor RNA. Different amounts of the mutant virus were added to equal aliquots of the sample containing an unknown amount of virus and RNA extraction and reverse transcriptase PCR (RT–PCR) were carried out in a manner that allowed for relatively precise quantitaion of the amount of viral RNA present in the sample.

MATERIALS AND METHODS

Cells

Cells (293) were obtained from ATCC and maintained in MEM medium containing 10% fetal calf serum. MT-2 cells were obtained from Dr. D. Richman through the National Institute of Allergy and Infectious Diseases (NIAID) AIDS research program and were maintained in RPMI 1640 medium containing 10% fetal calf serum.[17]

Generation of Mutant HIV-1 Virus

A well-conserved region between the primer-binding and gag initiation sites of HIV-1 was chosen for insertion.[18] The plasmid pNL4.3, an infectious molecular clone of HIV-1 obtained from Dr. M. Martin, was used as the starting material.[19] A mutant (VX-46) with the insert 5′-AGACATCTAGACGCGTCTAGACGCG-3′ at nucleotide position 715 of pNL4.3 was generated using standard procedures as described in Sambrook et al.[20] The HIV-1 nucleotide numbering system used is according to HIVNL4.3

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(GenBank accession number M19921). The VX-46 DNA was transfected into 293 cells by the calcium phosphate precipitation method.[20] Forty-eight hours after transfection, the culture fluid was taken and used to infect MT-2 cells. The mutant virus grew with similar kinetics as that of wild-type virus (V. Natarajan, unpubl.), and the cell-free virus was harvested, aliquoted, and stored at −70°C. Each aliquot was used once in internally controlled virion PCR (ICVPCR) experiments. The amount of virus in the culture supernatant was estimated by p24 antigen ELISA assay using Coulter Corp. (Hialeah, FL) reagents following the manufacturer's recommended protocol.

PCR Primers

The ICVPCR primers described in Table 1 were synthesized with an Applied Biosystems Inc. DNA synthesizer (Foster City, CA).

RNA Isolation and cDNA Synthesis

After informed consent was obtained, whole-blood samples were collected from HIV-1 seropositive patients in the presence of acid citrate dextrose as an anticoagulant. The plasma was separated by centrifugation and stored at −70°C until use. Aliquots (100 μl) of serial dilutions of the mutant virus VX-46 were added to 100 μl of patient's plasma, and RNA was extracted with 4.0 M guanidium thiocyanate and phenol in the presence of 180 mM sodium acetate as described earlier.[21] The RNA was precipitated by addition of an equal volume of isopropanol and 10 μg of carrier tRNA (Sigma Chemical Co.).

RNA was recovered by centrifugation, washed with cold 70% ethanol, dissolved in 8.0 μl of DNase buffer containing 50 mM Tris (pH 8.0), 5 mM $MgCl_2$, and 10 units of RNase-free DNase (Boehringer Mannheim Biochemicals), and incubated at 25°C for 30 min. The DNase was inactivated at 80°C for 10 min, and the RNA was used for cDNA synthesis using a cDNA cycle kit obtained from Invitrogen Corporation (San Diego, CA). Briefly, 2 μl of 100 mM methyl mercuric hydroxide was added to 8 μl of RNA, and after 5 min of incubation at room temperature, 2.5 μl of 0.7 M β-mercaptoethanol was added and the reaction was kept on ice. To this was added 4.0 μl of 5× RT buffer (0.5 M Tris at pH 8.3, 0.2 M KCl, 50 mM $MgCl_2$), 1.0 μl of RNase inhibitor, 1.0 μl of 25 mM of the dNTPs, 1.0 μl of primer ICVPCR-9 (40 pmoles), and 0.5 μl of AMV reverse transcriptase (5 units), and it was incubated at 42°C for 1 hr. The reaction was then heated to 95°C for 3 min and cooled on ice for 2 min, five more units of reverse transcriptase were added, and the incubation was continued for another hour at 42°C. Finally, the reaction was heated to 95°C for 3 min, and the cDNA was either used in the PCR or stored at −20°C.

TABLE 1 Oligonucleotide Deoxyribonucleotide Primer Sequences Used in PCR

Primer	Sequence	Position in HIVNL4.3
ICVPCR-9	5′-TCCCTGCTTGCCCATACTA-3′	890–908 (complementary)
ICVPCR-16	5′-ATCTCTCGACGCAGGACT-3′	681–698
ICVPCR-17	5′-GCTCTCGCACCCATCTCT-3′	786–803 (complementary)
ICVPCR-18	5′-ACTAGCGGAGGCTAGAAGGA-3′	765–784

PCR

Five microliters of cDNA was used in a reaction containing 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTPs, 25 pmoles of primers ICVPCR-16 and ICVPCR-17, and 2.5 units of *Taq* DNA polymerase (Perkin-Elmer Cetus) in a final volume of 50 μl. The samples were amplified in a Perkin-Elmer Cetus thermocycler (9600) with the following PCR cycle program: one cycle at 94°C for 60 sec, 55°C for 10 sec, and 72°C for 30 sec; 30 cycles at 94°C for 15 sec, 55°C for 30 sec, 72°C for 60 sec, and a final incubation at 72°C for 10 min. The samples were then stored at 4°C until analysis. The competitor plasmid (pA1) DNA used to estimate the amount of cDNA from VX-46 virus contained pNL4.3 nucleotide sequences from 501 to 1448.

Analysis of the PCR Product

Fifteen microliters of PCR product was hybridized with 5′-^{32}P-labeled oligonucleotide ICVPCR-18, separated in a 10% polyacrylamide gel, and autoradiographed as described earlier.[22] The amount of radioactivity present in each band was quantitated using either the Molecular Dynamics PhosphorImager (Sunnyvale, CA) or Fuji Medical Systems BAS1000 (Stamford, CT) by exposing the gel to a storage Phosphor screen as described by Johnston et al.[23]

RESULTS AND DISCUSSION

Estimation of RNA Present in a VX-46 Virus Preparation

RNA was isolated from aliquots of VX-46 virus containing 100 pg, 25 pg, or 500 fg of p24 antigen, and cDNA was synthesized using standard conditions. To establish and standardize the assay, cDNA from VX-46 virus was PCR-amplified in the presence of different amounts of plasmid DNA (pA1) containing pNL4.3 nucleotide sequences from 501 to 1448. As shown in Figure 1, the primers selected gave the predicted 123- and 148-bp DNA PCR products from the wild-type and mutant viral sequences, respectively. In some experiments additional minor bands were seen. Most likely these are heteroduplexes formed between the two expected bands. Also,

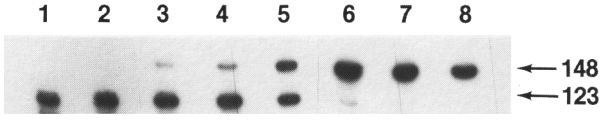

FIGURE 1 Use of cDNA synthesized from mutant virus (VX-46) RNA as competitor in PCR. RNA isolated from the mutant virus containing 100 pg of p24 antigen was used to synthesize cDNA in a 60-μl reaction. Two microliters of the cDNA was used in the PCR containing various amounts of competitor wild-type DNA (pA1) containing HIVNL4.3 sequences from 501 to 1448. The PCR was carried out, and the products were hybridized with a ^{32}P-labeled probe and autoradiographed as described in Materials and Methods. The competitor DNAs present in the reactions were 10^6 (lane *1*); 5×10^5 (lane *2*); 10^5 (lane *3*); 5×10^4 (lane *4*); 10^4 (lane *5*); 10^3 (lane *6*); 10^2 (lane *7*); and 0 (lane *8*) copies. The sizes shown (*right*) were the PCR products in base pairs from mutant and wild-type sequences, respectively.

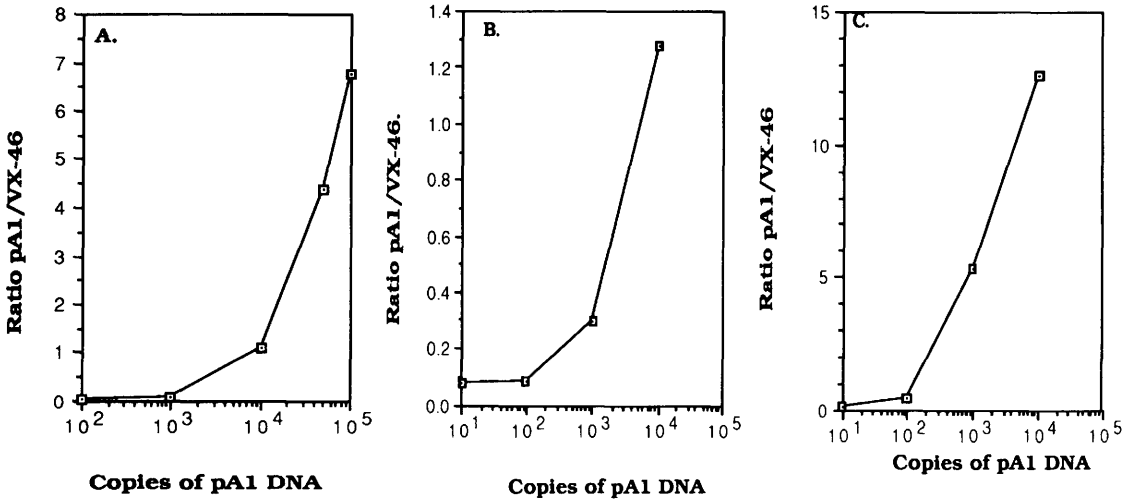

FIGURE 2 Estimation of the RNA isolated from the mutant virus VX-46. The amount of radioactivity present in bands corresponding to wild-type and mutant PCR products was estimated by exposing it to a storage Phosphor screen and quantitated using a Molecular Dynamics PhosphorImager.[23] The ratio between the amount of radioactivity present in wild-type (pA1) and mutant (VX-46) DNA bands was plotted against the amount of wild-type DNA used in the PCR. The mutant viral RNA was isolated from virus containing 100 pg (*A*), 25 pg (*B*), and 500 fg (*C*) of p24 antigen, respectively. The data derived from Fig. 1 are shown in *A*. The RNA isolated from virus containing 25 pg (*B*) and 500 fg (*C*) of p24 was used to synthesize cDNA in a 40-μl reaction, and 3 μl of cDNA was used in competitive PCR. Based on the method described by Menzo et al.,[16] we calculate that 2 μl of cDNA in *A* has 9500, 3 μl of cDNA in *B* has 5200, and 3 μl of cDNA in *C* has 120 copies, respectively.

the unused excess primer from the cDNA synthesis step can participate in the PCR reaction and generate a longer product (seen in Figs. 3 and 4, below). These bands do not interfere with accurate quantitation because the quantitation by this method is based on relative levels of bands from target and competitive templates and not on the absolute amounts.[24] The amounts of radioactivity in the specific bands were determined as described in Materials and methods. The ratios between the amounts of radioactivity present in wild-type and mutant DNA bands were plotted as described earlier.[15] On the basis of these data (Fig. 2), we calculated that 285,000, 69,300, and 1600 copies of RNA were isolated from virus containing 100 pg, 25 pg, and 500 fg of p24 antigen, respectively.

Based on the mass of an HIV-1 particle, a virus preparation with 100 pg of p24 would be calculated to contain 1 million HIV particles,[25,26] or 2 million copies of RNA. Thus, the copy numbers determined in our experiment represent ~15% of the theoretical value. This reflects loss of RNA during extraction and lower than the theoretical yields in the preparation of cDNA. However, the quantitation by competitive PCR relies on the relative levels of wild-type and mutant templates, rather than the absolute amount. Therefore, once the determination of the amount of RNA in a mutant virus preparation is made, the recovery of RNA in each ICVPCR experiment does not affect the outcome of the results.

ICVPCR to Determine the Level of HIV-1 RNA in Plasma

Using this standardized VX-46 virus as the competitor during the RNA isolation,

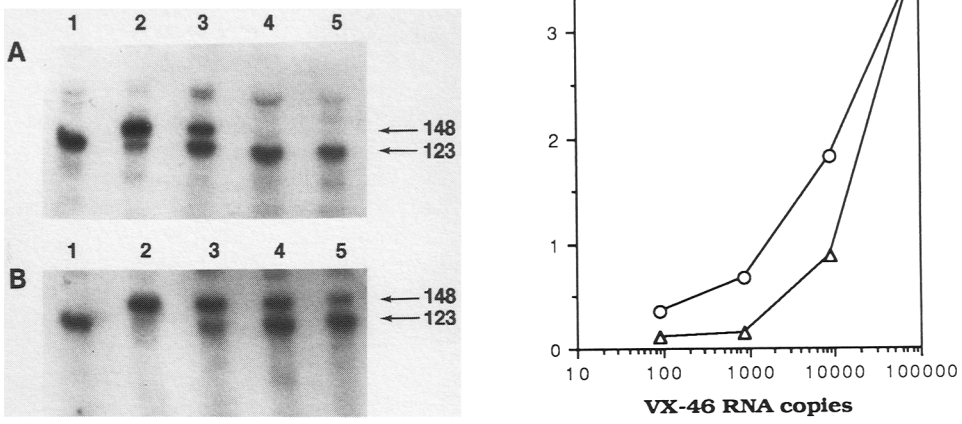

FIGURE 3 ICVPCR for the estimation of HIV-1 RNA in patient's plasma. To 100 μl aliquots of patient's plasma, different dilutions of mutant virus (VX-46) containing 0 (lane *1*), 30 (lane *2*), 3 (lane *3*), 0.3 (lane *4*), and 0.03 (lane *5*) pg of p24 antigen were added. RNA isolation, cDNA synthesis, and PCR were carried out as described in Materials and Methods. The size of DNA products from mutant (148) and wild type (123) are shown (*right*). (*A*, *B*) Plasma from two different patients. (*C*) Estimation of HIV-1 RNA in patient's plasma. The radioactivity present in each band was quantitated using a Molecular Dynamics PhosphorImager. The ratio between the radioactivity present in mutant and patient DNA bands was plotted against the amount of mutant viral RNA added to the patient's plasma during RNA isolation. The mutant viral RNA copy numbers were calculated using the average value (2900 copies of RNA per picogram of p24 antigen) obtained with the data shown in Fig. 2. Patient 1 (△) has 8800 copies; patient 2 (○) has 1600 copies of RNA in 100 μl of plasma.

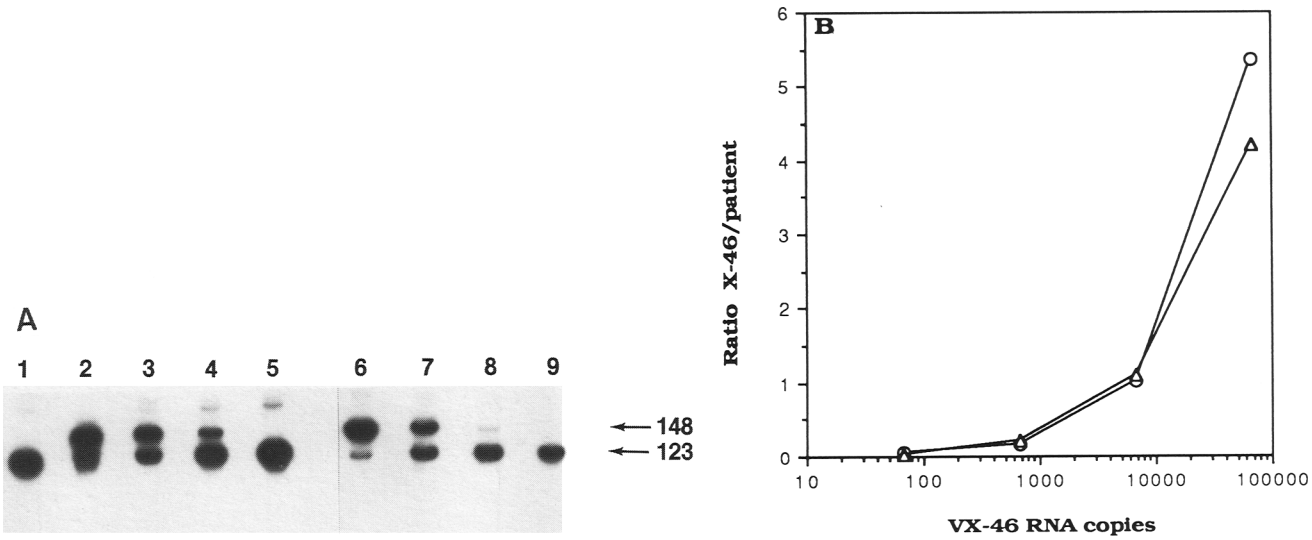

FIGURE 4 Reproducibility of ICVPCR. (*A*) To 100 μl aliquots of patient's plasma, different dilutions of mutant virus (VX-46) containing 0 (lane *1*), 24 (lanes *2,6*), 2.4 (lanes *3,7*), 0.24 (lanes *4,8*), and 0.024 (lane *5,9*) pg of p24 antigen were added. RNA isolation, cDNA synthesis, PCR, and gel analysis for lanes *1–5* and *6–9* were carried out by two separate experiments on different days. The sizes of DNA products from mutant (148) and wild type (123) are shown on the *right*. (*B*) The radioactivity present in each band was quantitated and plotted as described in Materials and Methods. The data for △ were from lanes *2–5*; that for ○ were from lanes *6–9*.

the amount of HIV-1 viral RNA in a patient's plasma was estimated. The results obtained with two different plasma samples are shown in Figure 3. The amount of radioactivity present in each band was estimated, and the ratio between the amount of radioactivity present in the mutant and wild-type (patient) DNA bands was plotted against the input mutant viral RNA. Using this technique, the amounts of viral RNA present in the samples were determined to be 88,000 and 16,000 copies of RNAs per milliliter of plasma from patients 1 and 2, respectively (Fig. 3C).

The reproducibility of the ICVPCR method was then assessed. The amount of viral RNA in a plasma sample was estimated by performing the entire assay on two different days. The amount of RNA by these two estimates were 60,000 and 68,000 copies per milliliter of plasma (Fig. 4), demonstrating that the ICVPCR method is reliably reproducible.

Competitive RNA PCR has been used successfully for the estimation of the levels of HIV-1 viral RNA present in patient samples.[27,28] In this procedure, the ratio of the amplified products is affected equally by the factors that influence the PCR. However, it lacks a control for the RNA purification step. It has been estimated that, on average, 36% of the RNA sample can be lost as a result of the extraction procedures used.[28] To account for the variable loss of RNA when gene expression is studied using RNA extracted from cells, the RNA expressed by the gene under study is often compared with another RNA species that is constitutively expressed.[29,30] However, such a control RNA is not available in plasma. By adopting the modified method described in this report, the mutant virion RNA can serve both as a control for the RNA extraction procedure and as a competitive RNA template in the PCR.

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