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QUANTIFICATION OF HIV GAG RNA USING REAL TIME REVERSE TRANSCRIPTASE PCR

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1. ABSTRACT

Quantification of HIV-1 is important to quantify risk for disease progression as well as for acquiring infection associated with drug abuse. Prior quantification methods include immune and enzymatic procedures, e.g., quantifying HIV-1 p24 protein by ELISA and the Reverse Transcriptase by enzymatic assay. Improved quantification of HIV-1 RNA and cDNA was established using PCR. This paper describes a real-time PCR technique using the Applied Biosystems 5700 Sequence Detection System and Taqman reverse transcriptase PCR. We initially standardized the PCR method using ribosomal-RNA to obtain relative quantification. Pure gag RNA was used for standard curves, controls, and to obtain absolute RNA quantification. Pure HIV gag RNA was produced by T7-directed transcription of the plasmid pWISP98-85. Detailed statistical analyses describe using absolute standard curves, and intraassay and interassay coefficients of variation to validate the methods. The presented method is highly reproducible and the assay's performance is comparable to prior assays. The assay is validated with an 8-log range down to 80 copies.

2. INTRODUCTION

Drug abuse and the HIV/AIDS are intersecting epidemics with global implications (1). Each fuels the other and places a constraint on the abrogation of the other. The *sine qua non* for the evaluation of HIV-1 risk to develop AIDS-defining illness for any individual patient is quantification of plasma HIV-1. Quantification of HIV-1 in CSF is indicative of HIV-1 brain infection (reviewed in 2). The level of virus (virus load) of each infected individual is a key variable to predict transmission through both sexual contact and the use of drug paraphernalia (3, 4, 5, 6, 7, 8, 9, 10, 11). HIV-1 is quantified in different fractions of blood including whole blood, plasma, serum, peripheral blood mononuclear cells (PBMNCs) and platelets (5, 11, 12, 13, 14) for risk for spread of HIV-1.

The illicit injection drug use (IDU) epidemic of the latter part of the 20th century coincides with the advent of AIDS (15). Risk for HIV-1 infection is incurred by behaviors including re-use of drug abuse utensils and paraphernalia such as needle/syringes [n/s], cottons, cookers, and wash-waters (predominantly used for injection of cocaine and opiate illicit drugs) (9, 10, 17, 18). HIV-1 is quantified in contaminated drug paraphernalia as risk vectors of transmission. Additional risks are unprotected sex and exchanging sex for money or drugs associated with illicit drug use such as crack cocaine-smoking (1, 6, 7, 8, 16, 20, 21). Use of these drugs has deleterious effects on the immune system in both animal models and human studies (22, 23, 24, 25, 26). Injection drug use behaviors, through the sharing of infected needles and other paraphernalia continue to be the highest HIV risk behavior in many areas and seroprevalence rates vary worldwide (27, 28, 29, 30). The seroprevalence rate rose to nearly 1% prevalence in the 15- to 49-year-old populations of Lao PDR, Myanmar, and Thailand (the Golden Triangle) and increased up to 80% among IDUs in many risk areas within fewer than 10 years (15). IDU behavior continues as the highest risk for HIV infection in many places (27, 28, 29, 30).

During the last decade, there have been advances in the detection and quantification of HIV. Initially, enzyme linked assay (EIA) or enzyme linked immunosorbent assay (ELISA) and enzymatic methods were used to detect HIV-1. For example, p24 ELISA and reverse transcriptase enzymatic measures (12) were used earlier in the epidemic. With the advent of polymerase chain reaction (PCR), application of this method to HIV resulted in rapid technological advances. Several studies

suggested that EIA was more sensitive than Western Blot to detect HIV-1 antibodies and PCR to detect proviral DNA in n/s (31, 32). Kwok and colleagues, and many others made advances from initial application of PCR to HIV detection to the quantification of HIV DNA and then RNA sequences (33, 34). HIV gag is generally the gene of choice due to its lower sequence heterogeneity (35, 36).

Several types of PCR methods were developed. In 1993, Nakamura *et al* (37) amplified and detected a single molecule of HIV-1 RNA utilizing a two-tube nested reverse transcriptase PCR procedure. In 1994, Lin *et al*, (38) compared quantification of HIV-1 RNA in plasma in a Multicenter evaluation of six methods that included the Roche Molecular Systems using single enzyme RT-PCR, the Chiron branched chain (bDNA), and phosphor-imaging techniques for analysis of the PCR amplicons. Assay factors of importance in the evaluation of these techniques included specificity, sensitivity, precision, sample volume, throughput, cost, ease of performance, and turnaround time. bDNA used alkaline phosphatase detection method and Roche uses horseradish peroxidase detection method. The two assays gave comparable results for HIV-1 detection (38, 39, 40) promulgated a one-tube quantitative HIV-1 RNA nucleic acid sequence based amplification (NASBA) assay using electroluminescent-labeled probes. This one-tube method achieved 5-log concentration range and demonstrated the value of non-radioisotopic methods for quantification of PCR amplicons. A novel real time PCR method devised to detect HIV-1 RNA and DNA based on a fluorogenic primer-probe adduct was developed called "scorpion." The probe hybridized to the extension of the adjoining primer intramolecularly (41). The assay had 10-copy viral template sensitivity and had a wider dynamic range (up to 10^8 copies) than prior end-point PCR assays. The Roche "Amplicor" PCR assay has a sensitivity of 400 copies and the Roche Ultrasensitive PCR assay has a sensitivity of 50 copies (14; Roche Kit Instruction Protocols). Eight tubes are required per specimen and these assays have a two-log range with internal and external controls. A radioactive PCR ^{32}P-labeled primer method was developed in which amplicons were separated by agarose gel electrophoresis and then scanned to quantify band intensities (42, 43). In addition, PCR was used to amplify HIV-1 gag and envelope genes followed by heteroduplex hybridization to distinguish among HIV-1 subtypes (44). Quantification of HIV-1 infection is important for the estimation of risk for HIV-1 contamination of drug abuse paraphernalia and the dynamics of spread of HIV infection of IDU subjects (9, 16).

A real time PCR assay was used for detection of HIV-1 at a sensitivity of 62 copies per microgram of RNA. The range was up to 50,000 copies per microgram (45). This method did not use the Primer Express method of Applied Biosystems. It relied on prior primers, probes, and PCR profile steps and did not have extensive statistical analysis or standardization of the assay. This method detected unspliced mRNA after prolonged antiretroviral therapy (45, 46).

We therefore devised an absolute real time Reverse Transcriptase (RT) PCR assay to quantify HIV-1_B gag with a wider range of detection, relying on Primer Express, and also with focus on statistical validation methods (47).

3. METHODS

3.1. Description and Preparation of Plasmid pWIS98-85

The plasmid, pWISP98-85, was obtained from the Research and Reference Reagent Program, Division of AIDS, NIAID, NIH and was originally produced by Dr. Wes Sundquist (48). Growth of the plasmid and RNA transcription were done according to Sundquist's procedures (48). A 699 bp insert containing the HIV-1 gag capsid coding sequence from pNL4-3 was isolated by PCR. The insert was cloned into the NdeI-BamHI site of pET11a (Novagen). The T7 promoter drives the pET expression system. The plasmid was grown in transformed DH5-α bacteria in LB (with Ampicillin) and stored in LB containing 20% glycerol (with Ampicillin). We used the QIAGEN QIAfilter Midi kit for plasmid DNA Extraction.

3.2. RNA transcription from pWIS98-85

Restriction Enzymes Nde I and Bam HI (New England Biolabs and Promega, Inc) were used to digest the plasmid pWIS98-85 according to the manufacturer instruction. One % Agarose Gel electrophoresis and Agilent Technology 2100 Bioanalyzer Isotacophoresis (according to the manufacturer instructions) were used to visualize the digest products. Agilent DNA 7500 Assay Kit chips were used. Linearized plasmid was purified using the QIAquick PCR purification Kit from QIAGEN and used as template for *in vitro* transcription (Megascript High Yield Transcription Kit, Ambion). DNAse followed by the phenol: chloroform-isoamyl alcohol method was used to purify the RNA product. We used Phase Lock Gel 1.5 ml from Eppendorf Corp., to enhance separation of the aqueous and organic phases. The Agilent Bioanalyzer was also used with the RNA 6000 Assay kit to check the quality of the RNA.

3.3. RNA extraction

QIAGEN QIAamp RNA Mini kits were used (according to manufacturer instructions) for total RNA extraction. The purified RNA samples were suspended in RNAse-free water, aliquoted, and stored at -80C for subsequent Real Time RT-PCR Assays.

3.4. Real Time PCR

We performed two types of Real Time PCR. The Ribosomal (18S) RNA control Reagent Kit from Applied Biosystems (AB) was used for rRNA quantification from which relative copy numbers of HIV-1 were obtained. Pure monodisperse gag RNA transcripts were produced from plasmid pWIS98-85 for absolute quantification of HIV-1 RNA. Invitrogen-Life Technologies synthesized the primers and Applied Biosystems, Inc synthesized the Vic/TAMRA Probe. The one-step Taqman assay was performed throughout the study precisely according to manufacturer instructions (AB). The cycle transition value (C_t) is produced as an output from the Applied Biosystems 5700 SDS Analyzer and is based on extrapolation to the threshold value (or baseline) of the fluorescence curve for

Figure 1. Agilent plasmid DNA. DNA Ladder and two preparations of linearized pWISP98-85 plasmid DNA, 6,652 bp, BamH1. DNA was analyzed by Agilent isotacophoresis as described in the text.

Figure 2. Agilent HIV gag RNA. RNA Ladder and two preparations of *in vitro* RNA synthesized from linearized pWISP98-85 plasmid DNA. T7 RNA polymerase used, 699 nt RNA transcript product. RNA was analyzed by Agilent isotacophoresis.

each well by the software. The threshold value is based on optimization of the correlation coefficient (r) of each standard curve performed according to manufacturer instructions (AB) for each plate.

For each HIV-1 experiment, pure gag RNA was serially diluted from 3,000 pg (3 ng) to 0.00003 pg (30 ag). The corresponding copy numbers were 7.59×10^{9} and 75.9, respectively. For each rRNA experiment, pure rRNA was serially diluted from 2,500 pg to 0.025 pg [18S Raji cell rRNA (Communication from Technical Services, ABI)]. The corresponding copy numbers were 2.36×10^{10} and 2.36×10^{5}, respectively. The serially diluted standards were used to generate the standard curve on each 96-well plate. Standards were serially diluted for every plate run. The same serially diluted solutions were also used as unknowns on the same plates. Every determination is always done in quadruplicate. In addition, for HIV-1 two interplate standards were diluted, aliquoted, cryopreserved at –80 ^{o}C, and run on every plate. These are labeled A2 and B2 and are 3.00 and 0.03 pg/reaction respectively. Thus, the general principle is that each HIV-1 assay plate has a fresh serial dilution of RNA (intra-assay standard) as well as a pre-diluted RNA that function as inter-assay standards.

3.5. Statistics

As stated above, standards were diluted for measurement and treated as unknowns. Every standard and unknown was measured in quadruplicate in every experiment (plate). In addition, two cryo-preserved (-85 ^{o}C) pre-diluted standards were assayed in four experiments. The data for assay validation included means of the four replicates of copy number as well as means of the four replicates of the cycle transition (C_t) number. Within each standard dilution the mean, standard error of the mean, 95% confidence limit, and coefficient of variation (100 x standard error/mean) were computed. The coefficient of variation is an appropriate measure in the present circumstances because it measures the "relative" error rather than the absolute error, which is an increasing function of copy number. Within-experiment repeatability was analyzed by computing and comparing the "within" and "between" variances and coefficients of variation for each of the standards.

4. RESULTS

The pWIS98-85 plasmid was linearized with restriction enzyme Bam HI. One percent agarose gel electrophoresis (data not shown) and Agilent Bioanalyzer Isotacophoresis demonstrated the size of the vector containing the gag CA region insert was 6,652 bp (shown in Figure 1). One ug of linearized DNA produced a yield of 85 ug of RNA. The HIV gag RNA produced was monodisperse and visualized by Agilent Bioanalyzer Isotacophoresis (Figure 2). Restriction enzymes Nde I and Bam HI were used to confirm the gag CA region insert in the plasmid pWIS98-85. One percent agarose gel electrophoresis and Agilent 2100 Bioanalyzer Isotacophoresis (data not shown) indicated the expected DNA fragment size (699 bp).

Table 1. Primers and Probe: HIV-1 gag (CA region)

Primers and Probe	Sequence 5'→ 3'
Forward Primer HIV-1 gag-1	AGTAAGAATGTATAGCCCTACCAGCAT
Reverse Primer HIV-1 gag-2	CTTAGAGTTTTATAGAACCGGTCTACATAGTC
TaqMan® Probe gag	CTGGACATAAGACAAGGACCAAAGGAACCC (VIC --- TAMRA)

The primer and probe sequences were produced using Applied Biosystems PC program, Primer Express as described in the text

Table 2. Assay and Validation of Copy Number, HIV-1 gag

	Input Copies	Mean C_t	SE C_t	95% CI C_t	CV C_t	Mean Copies	SE Copies	95% CI Copies	CV Copies
Standard 1[1]	7.59x10^{9}	9.46	.019	9.40-9.52	0.2	7.25 x10^{9}	1.14 x10^{8}	6.88-7.61 x10^{9}	1.6
Standard 2[1]	7.59x10^{8}	10.90	.144	10.44-11.35	1.3	7.46 x10^{8}	1.90 x10^{7}	6.85-8.06 x10^{8}	2.6
Standard 3[1]	7.59x10^{7}	18.06	.236	17.31-18.81	1.3	6.67 x10^{7}	2.13 x10^{6}	5.99-7.35 x10^{7}	3.2
Standard 4	7.59x10^{6}	19.79	.103	19.46-20.11	0.5	8.16 x10^{6}	2.57 x10^{5}	7.34-8.97 x10^{6}	3.2
Standard 5	7.59x10^{5}	23.25	.106	22.91-23.58	0.5	7.15 x10^{5}	7.03 x10^{3}	6.93-7.37 x10^{5}	1.0
Standard 6	7.59x10^{4}	26.78	..054	26.61-26.95	0.2	7.65 x10^{4}	2.43 x10^{3}	6.88- 8.42 x10^{4}	3.2
Standard 7	7.59x10^{3}	30.78	.215	30.09-31.46	0.7	6.90 x10^{3}	7.09 x10^{2}	6.68-7.13 x10^{3}	1.0
Standard 8	7.59x10^{2}	34.02	.086	33.74-34.29	0.3	7.10 x10^{2}	1.50 x10^{1}	6.61-7.57 x10^{2}	2.1
Standard 9	7.59x10^{1}	37.45	.203	36.80-38.09	0.5	6.62 x10^{1}	1.54x10^{0}	6.13-7.11 x10^{1}	2.3
A2	7.59x10^{6}	19.50	.143	19.14-19.87	0.7	7.77 x10^{6}	6.19x10^{5}	6.18 – 9.37 x10^{6}	8.0
B2	7.59x10^{4}	26.99	.203	26.47-27.51	0.8	6.51 x10^{4}	2.23 x10^{3}	5.94-7.09 x10^{4}	3.4

[1]Calculations done on four replicates within only one experiment each

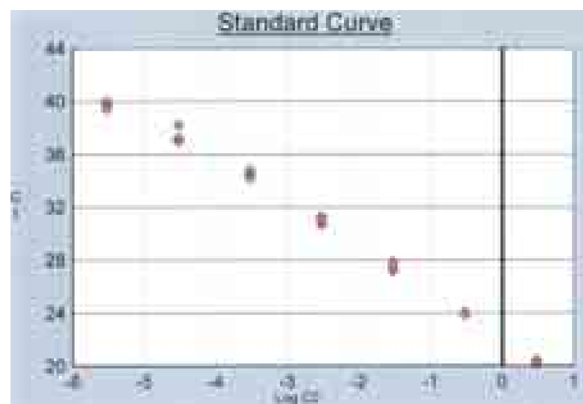

Figure 3. Standard Log-linear regression HIV gag RNA C_t vs. log concentration HIV RNA plotted by computer-Applied Biosystems 5700 set-up. The one-tube Taqman kit was used for this assay with probe and primers shown in Table 1.

The primer and probe sequences used in our study were produced by AB Primer Express and are shown in Table 1. They were optimized for the PCR profile used by the AB 5700 (Applied Biosystems Inc, manufacturer's PCR kit profile instructions). The one-tube Taqman kits were used to analyze HIV-1_B gag RNA and rRNA. C_t vs. log concentration RNA assay was plotted by the computer-Applied Biosystems 5700 set-up.

For example, the pure HIV-1 gag RNA (Figure 3) C_t vs. log concentration gag RNA was plotted by the computer-Applied Biosystems 5700 machine. Standard log-linear regression is shown. The slope is -3.30 and the correlation coefficient is -0.998. The efficiency of the amplification is also 2.0. The efficiencies of amplification for all the experiments varied between 1.97 and 2.0.

The results of the HIV real time RT PCR (for the gag CA region) are presented in Table 2. For each experiment, pure gag RNA was serially diluted from 3000 pg (3 nanog) to 0.00003 pg (30 attog). The serially diluted standards were used to generate the standard curve on each 96-well plate. Standards were serially diluted for every PCR plate run. The same serially diluted solutions were also used as unknowns on the same plates. Every determination is always in done in quadruplicate. In addition, two interplate standards were diluted, aliquoted, cryopreserved at –80 ^{o}C, and run on every plate. Table 2 shows the mean, standard error of the mean (SE), 95% confidence interval (95% CI), and coefficient of variation (CV) for copies/reaction (computed from the pg/dilution) and cycle transition value (C_t). The analysis of the C_t values reflects uniformly small instrumental error over a broad (6-8 log) dilution range. The CVs for Copy Number, which reflect technique as well as instrumental error, are larger, but do not depend on copy number. Moreover, the CVs are all under 10%, which is the generally accepted laboratory quality control standard.

To assess within-experiment variability, two approaches were taken. The first approach was to compute the within-experiment CV for the four replicates for each of the four experiments per standard, and then to compute the average and standard error for each standard. The results are shown in Table 3. It is clear that among the replicates within an experiment, the experimental variation is small and that the relative variation does not depend on the copy numbers in the reaction. The second approach was to compute the ratio of the within-experiment variance to the between-experiment variance for each standard and then average these ratios across standards. For the C_t observations the ratio of the within to between variances was 0.78 ± 0.46, and for the copy number observations the ratio was 0.53 ± 0.24. As was expected, the technique error is smaller than the preparation error.

Table 3. Within Experiment Coefficient of Variation (Four replicates, four experiments each), HIV gag

		CV of C_t Determinations		CV of Copy Determinations	
	Input Copies	**Mean**	**SE**	**Mean**	**SE**
Standard 4	7.59×10^6	0.377	0.188	3.347	0.143
Standard 5	7.59×10^5	0.095	0.047	2.075	0.094
Standard 6	7.59×10^4	0.189	0.094	4.225	0.807
Standard 7	7.59×10^3	0.194	0.457	1.575	0.283
Standard 8	7.59×10^2	0.057	0.028	3.350	2.120
Standard 9	7.59×10^1	0.650	0.325	2.775	0.626
A2[1]	7.59×10^6	0.267	0.049	2.93	0.525
B2[1]	7.59×10^4	0.283	0.083	4.02	1.77

[1]Cryo-preserved (-85 °C) pre-diluted samples

Table 4. Assay and Validation of Copy Number, rRNA

	Input Copies	Mean C_t	SE C_t	95% CI C_t	CV C_t	Mean Copies	SE Copies	95% CI Copies	CV Copies
Standard 1	2.36×10^{10}	15.53	0.106	15.07-15.99	0.7	2.20×10^{10}	5.19×10^7	$2.17\text{-}2.22 \times 10^{10}$	0.2
Standard 2[1]	2.36×10^9	17.21	0.134	16.80-17.60	0.8	2.32×10^9	3.37×10^7	$2.22\text{-}2.41 \times 10^9$	1.5
Standard 3	2.36×10^8	21.45	0.548	19.09-23.80	2.6	2.25×10^8	2.25×10^6	$2.16\text{-}2.35 \times 10^8$	1.0
Standard 4	2.36×10^7	25.11	0.320	23.74-26.49	1.3	2.20×10^7	8.63×10^5	$1.82\text{-}2.57 \times 10^7$	3.9
Standard 5	2.36×10^6	28.57	0.144	27.95-29.19	0.5	2.18×10^6	7.09×10^3	$2.15\text{-}2.21 \times 10^6$	0.3
Standard 6	2.36×10^5	31.20	0.159	30.52-31.89	0.5	2.20×10^5	5.56×10^3	$1.96\text{-}2.44 \times 10^5$	2.5

[1]Only one experiment of four repeats, calculation done on repeats. The others are 2 experiments with 4 repeats each, calculations done on mean values

The results of the rRNA real time RT PCR are presented in Table 4. Two parameters are shown as for Table 2: rRNA copy number and C_t. Assay validation is also described using mean, standard error of the mean (SE), 95% CI, and CV as in Table 2 for HIV-1 gag. The results are very similar as those found for HIV-gag. The instrumental error is uniformly small and does not depend on dilution, whereas the relative copy error is somewhat larger but is independent of dilution. A comparable study was performed for cloned HIV-1_B DNA and similar results obtained (data not shown).

5. DISCUSSION

This paper presents and validates a highly reproducible and accurate assay for the real time quantification of HIV-1_B RNA molecules (gag RNA). A one-tube real time RT PCR method based on the Applied Biosystems Taqman fluorescent probe technology was utilized. This assay is linear across eight logs, takes 4 hours to perform, and can be used to analyze 14-16 specimens in quadruplicate per 96-well plate. Our performance of the assay produced extremely low CVs for RNA copy number and C_t for intra-assay and inter-assay variations. The assay shows lower than expected CVs (based on prior assays in the literature). Standards 4-9 are routinely used for the assay because they have a sufficient range for use with paraphernalia specimens (P. Shapshak, R. Duncan, and JB Page, unpublished observations, 2003).

Published studies show use of real time PCR that are based on relative quantification with respect to a housekeeping gene. The study presented here provides a more detailed statistical analysis of the reliability of the range and sensitivity of the assay than previously provided in the literature and is based on absolute quantification using a pure HIV-1 gag RNA standard. For example, using the "scorpion" probe mentioned above, Saha *et al* (41) produced interassay CVs of 1.66% to 45.68% and interassay CVs of 5.17% to 19.27%. Our CVs were constricted compared with published studies. HIV-1 replication was assessed in infected cells *in vitro* using multiple step-TaqMan RT relative-PCR (49). This assay was based on Applied Biosystems technology as previously described (50). Standard curves were based on gel-purified PCR cDNA products for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and HIV gag. HIV gag quantification was set at 100-copy sensitivity based on the relative gag and GADPH values. Similar studies have been accomplished for HIV-1 DNA demonstrating the value and utility of real time PCR (51).

Real-time quantitative PCR method was developed in two studies to measure RNA from the closely related virus, HIV-2 including strain subtypes A and B (52, 53). In the first study (52), the sensitivity of the HIV-1 combined assay for the two subtypes was 250 copies/ml using the Roche light cycler. This assay had a 3-log range. At 10^5 copies, the interassay variation was 1.78% and intra-assay variation was 1.04%. The second study (53) had a sensitivity of 100 copies based on electron microscopy to count virus particles and used the multi-step TaqMan AB system. The intra-assay variability for copy number and C_t was 2.5% at 10^4 copies. The intra-assay variability was 7.5% for C_t at 100 copies, the limit of detection. This assay had a four-log range.

There is a need for international standards that allow comparison of virus load among different assays and studies (54). This will be helpful in integrating the information obtained by TaqMan and light-cycler real time PCR assays.

Towards this end, the current study presents results using HIV-1 RNA standards for absolute quantification methods. Results are validated based on extensive error analysis including intra-assay and inter-assay standards. This is important in treatment of patients and in the drug-abuse field because of the need to quantify HIV risk through analysis of plasma, n/s, and other paraphernalia employed by illicit drug injection users.

Prior studies used several methods to evaluate the presence of HIV-1 risk in n/s and other paraphernalia. HIV-1 gag p24 was quantified in culture supernates after exposing HIV-1 preparation to several conditions in the laboratory simulating time and temperature in risk locale/shooting galleries (9, 18, 55, 56) and PCR (including the Roche Amplicor assay) was used to quantify HIV-1 RNA (16). Similarly, immune methods (Western Blot and ELISA) have been used to assess the HIV-status of subjects in epidemiological/ethnic studies (3, 57).

In the absolute quantification assay presented here, standards 4-9 are routinely used for the assay because they have a sufficient range for use with patient and drug paraphernalia specimens (data not shown). The sensitivity using quadruplicate measurements is 80 copies/reaction. The use of the real time PCR assay described here will provide a more sensitive and standardized method for HIV-1 RNA detection and quantification (47). The assay is similarly applicable to quantification of HIV-1 DNA and we have done so (data not shown). We have commenced application of this methodology to the quantification of HIV-1 RNA and DNA in n/s and other drug abuse paraphernalia.

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