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Post-extraction stabilization of HIV viral RNA for quantitative molecular tests

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Abstract

Two approaches to stabilize viral nucleic acid in processed clinical specimens were evaluated. HIV-1 RNA extracted from clinical specimens was stabilized in a dry matrix in a commercial product (RNAstable, Biomatrix, San Diego, CA, USA) and in a reverse-transcription reaction mixture in liquid form as cDNA. As few as 145 HIV-1 genome copies of viral RNA are reliably stabilized by RNAstable at 45°C for 92 days and in the cDNA format at 45°C for 7 days as determined by real-time PCR. With RNAstable the R^2 at days 1, 7, and 92 were 0.888, 0.871, and 0.943 when compared to baseline viral load values. The cDNA generated from the same clinical specimens was highly stable with an R^2 value of 0.762 when comparing viral load determinations at day 7 to baseline values. In conclusion viral RNA stabilized in a dry RNAstable matrix is highly stable for long periods of time at high temperatures across a substantial dynamic range. Viral RNA signal can also be stabilized in liquid in the form of cDNA for limited periods of time. Methods that reduce reliance on the cold chain and preserve specimen integrity are critical for extending the reach of molecular testing to low-resource settings. Products based on anhydrobiosis, such as the RNAstable should be evaluated further to support viral pathogen diagnosis.

Keywords

RNA virus; RNA stability; molecular diagnosis; viral load; point-of-care

1. Introduction

Ensuring specimen integrity remains a logistical constraint on molecular testing for RNA viruses (Dineva, et al., 2007; Puren, et al., 2010). Dried blood spots (DBS) and dried plasma spots (DPS) provide an effective means to preserve viral RNA in preprocessed clinical specimens and remove the requirement of the cold chain for significant periods of time (Andreotti, et al., 2010; de Vries, et al., 2009; de Crignis, et al., 2010; Leelawiwat, et al., 2009; Monleau, et al., 2009). As such, they represent methods that extend the reach of centralized testing systems particularly in settings with limited resources.

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DBS and DPS have been shown to be useful means for specimen transportation and preservation for several RNA viruses (Chibo, et al. 2005; Choi, et al., 2009; De Swart, et al., 2001; de Crignis, et al., 2010; Desbois, et al., 2009; Katz, et al., 2002; Lenselink, et al., 2009). They have been shown to support quantitative HIV viral load testing for monitoring patients on antiretroviral therapy (ART) (Andreotti, et al., 2010; Garrido, et al., 2009; Johannessen, et al., 2009a; Leelawiwat, et al., 2009; Lofgren, et al., 2009; Marconi, et al., 2009; Mbida, et al., 2009; Mehta, et al., 2009; Reigadas, et al., 2009; Viljoen, et al., 2010). However, DBS and DPS place constraints on the lower limit of detection (LOD) attainable and the dynamic range over which there is adequate quantitative discrimination (Andreotti, et al., 2010; Johannessen, et al., 2009a; Johannessen, et al., 2009b; Lofgren, et al., 2009). A commercial product ViveST™ (ViveBio, Lawrenceville, GA, USA) allows stabilization of much larger specimen volumes at ambient temperature, and thus supports quantitative tests with low LODs and accurate dynamic ranges (Lloyd, et al., 2009; Zaroni, et al., 2010). These methods support the stabilization of viral RNA in the clinical specimen matrix.

There are far fewer options to stabilize viral nucleic acid that has already been extracted from its clinical matrix. As viral load testing for patients on ART is implemented more widely and methodologies that support more decentralized testing systems emerge, the ability to ship and store specimens without reliance on the cold chain will become more critical. Furthermore, methods that stabilize extracted nucleic acids could potentially support emerging point-of-care diagnosis if the same method is applicable to stabilizing controls. Recently a new generation of products, such as GenTegra DNA and RNA (GenVault-IntegenX, Pleasanton, CA, USA), and Biomatrix DNASTable (also available as QIA-safe products through Qiagen, Valencia, CA, USA) and RNASTable (Biomatrix, San Diego, CA, USA) have emerged that claim to be able to stabilize DNA and RNA in dry format without requiring refrigeration (Hernandez et al., 2009; Wan et al., 2009; Fripiat et al., 2011). Some of these are based on the principle for anhydrobiosis a biological process employed by multicellular organisms that are able to survive at close to complete dehydration. This class of products could extend the shelf lives of testing kits and lower shipment costs, additionally they could also support stabilization of controls. However to date there has been no evaluation of these products for their application to viral nucleic acid. The capacity of a commercial product, RNASTable, to preserve viral RNA in a dry matrix for delayed assessment in a quantitative molecular assay was evaluated. The stability of viral RNA signal in the form of cDNA in liquid was also evaluated.

2. Methods

2.1 Specimen collection and standards

2.1.1 Clinical specimens/Harborview-University of Washington (Seattle, WA, USA)—Frozen HIV-1-positive human plasma was obtained from the University of Washington's HIV Specimen Repository. Samples were transported to PATH on dry ice and immediately transferred to freezer storage at -80°C . Using viral load specimens supplied by the repository, samples were selected by non-research staff to cover a broad distribution of viral loads ranging from 83 to 3.0×10^5 copies/mL. The repository determined the viral loads of individual samples using a variety of assays between August 2006 and January 2009. Specimens were reassessed at PATH for baseline viral loads in order to eliminate variation caused by differing assays and specimen storage times. Research staff members were blinded to all viral load data supplied by the repository until all samples had been processed. Use of the samples for this purpose had been approved by the PATH ethical review board in compliance with the University of Washington's HIV Specimen Repository policy for accessing archived specimens.

2.1.2 Acrometrix Optiquant—HIV-1 RNA standards (OptiQuant, P/N 94-2013, AcroMetrix, Benicia, CA, USA) consisted of heat-inactivated whole virion diluted in pooled EDTA-treated normal human plasma. The HIV-1 RNA concentrations in this panel ranged from 1×10^7 to 1×10^2 IU/mL (corresponding to 5.8×10^6 to 58 c/mL, as determined by Roche Molecular Systems, Pleasanton, CA, USA). All panel members were quantified by the manufacturer via the COBAS® Amplicor HIV-1 Monitor v1.5 UltraSensitive Assay (Roche Molecular Systems, Inc., Pleasanton, CA, USA) prior to shipment. Standards were shipped overnight on dry ice, which was still present upon arrival. All standards were stored at -80°C and thawed at room temperature immediately prior to usage.

2.2 RNA extraction and stabilization

All extractions were performed on 200 μL of plasma using the QIAamp® Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) as recommended by the manufacturer. RNA captured on a column was eluted with 60 μL of RNase/DNase-free water.

2.2.1 Specimen stabilization on RNastable—For each sample, 25 μL of extraction eluate was applied to the bottom of a 1.5-mL tube of RNastable. Samples were then dried down for two hours at ambient temperature in a centrifugal vacuum concentrator (Vacufuge® plus, Eppendorf, Hamburg, Germany). Tubes were immediately recapped, sealed in a foil pouch with desiccant, and stored at 45°C until reaching their testing date. On the day of testing, RNA was resuspended in 25 μL of DNase/RNase-free water. Resuspensions were incubated at room temperature for 15 to 25 minutes after which 20 μL of the resuspension was immediately evaluated via the previously described HIV-1 G2 LTR RT-PCR assay (Rouet, et al., 2005; Rouet, et al., 2010). Brief vortexing was required to disperse the resuspension evenly within the RT-PCR tube.

2.2.2 Specimen stabilization by cDNA preparation—Extracted RNA was reverse transcribed at 48°C for 30 minutes followed by incubation at 95°C for 10 minutes using the G2 LTR assay conditions. All reactions had a total volume of 100 μL with 20 μL being extraction eluate (or DNase/RNase-free water for negative process controls). In order to minimize volume loss due to evaporation, cDNA reactions were transferred from their original reaction tubes into 1.5-mL lock-top eppendorf tubes for storage in the dark at 45°C for 7 days.

2.3 Viral load determination

2.3.1 Amplification—All reverse transcriptions and RT-PCR utilized the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) for thermocycling and fluorescent detection and used the G2 LTR RT-PCR assay conditions (Rouet, et al., 2010). Viral load determinations were made using 100- μL total reaction volumes including 20 μL of extraction eluate or resuspended RNastable preparations (rehydrated in 25 μL DNase/RNase-free water). Prior to thermocycling, the entire volume of each cDNA preparation was supplemented with 2 μL of SuperScript® III/Pt Taq (Invitrogen, Grand Island, NY, USA), and 2 μL of a G2 LTR RT-PCR primers and probe mix (10 μM each). All sample types (including samples stabilized as cDNA) underwent the same thermocycling profile: 30 minutes at 48°C , 10 minutes at 95°C , followed by 50 cycles of 15 seconds at 95°C , and 1 minute at 60°C .

Standard curves were run on each day, along with four negative PCR controls using DNase/RNase-free water. Standard curves were constructed by first extracting a single 1×10^7 IU/mL standard (1.7 IU/copy as determined by Roche Molecular Systems, Inc., Pleasanton, CA, USA), followed by 10-fold dilutions of extraction eluate in DNase/RNase-free water.

Standard curves were constructed using duplicate 100 μ L RT-PCR at log 7, 6, 5, 4, and 3 IU/mL (5.8×10^6 to 5.8×10^2 copies/mL).

2.3.2 Quantification—Standard curve validity was determined using the following criteria. All standard curve RT-PCR down to concentrations of 1×10^4 IU/mL needed to be positive. At least one out of two 1×10^3 IU/mL RT-PCR needed to be detectable. All standard curves had R^2 values >0.950 . Samples detected below the 500 c/mL cutoff were considered negative for the given RNA preparation and time point.

2.4 Specimen stabilization study

HIV-1 RNA was extracted as described in section 2.2 from retrospective plasma specimens (stored at -80°C) collected from HIV patients. After evaluation of initial extractions (Day 0) from plasma (stored at -80°C), stabilization methods were assessed after incubation at 45°C for 1, 7, and 92 days. 45°C was selected as an extreme thermal stress condition under which to test the different stability formulations. The Arrhenius equation allows extrapolation of temperature dependence on decay reaction rates and is used as the basis for accelerated stability studies (Kirkwood, 1977, Kirkwood, 1984). Based on the Arrhenius equation, these durations are equivalent to storage at 25°C for 4, 28, and 368 days respectively. The data presented does not permit extrapolation of stability beyond this. Viral RNA was stabilized dried down in RNastable or in liquid form as cDNA as described in sections 2.2.1 and 2.2.2 respectively.

2.5 Timeline

2.5.1 Day 0—Two extractions were performed for each clinical sample. Eluates from these duplicate extractions were pooled to minimize the effects of inter-extraction variability and to maximize comparability of stabilization techniques. RNA for each sample was then stabilized in two tubes of RNastable (to be tested on Day 1 and Day 7), and in one reverse transcription reaction to cDNA (to be tested on Day 7). Negative controls were prepared using water in place of extraction eluate for both RNastable and cDNA stabilization techniques. Extraction eluate pools were also used to establish viral load baselines for all comparisons with later time points (Figure 1).

2.5.2 Day 1—One of each RNastable preparation from Day 0 was rehydrated for each clinical sample and negative control. Viral loads were immediately evaluated by RT-PCR (Figure 1).

2.5.3 Day 7—One of each RNastable preparation from Day 0 was rehydrated for each clinical sample along with negative controls. Aliquots of the same clinical plasma samples (stored at -80°C) were also freshly extracted on Day 7 to evaluate day-to-day process and assay consistency. Viral loads for fresh extractions, cDNA, and rehydrated RNastable preparations (prepared on Day 0, and stored at 45°C) were all evaluated side by side with RT-PCR (Figure 1).

2.5.4 Day 92—Additional extractions were performed for a subset of samples and stabilized in RNastable and stored at 45°C . These were rehydrated and evaluated 92 days later.

2.6 RNastable process controls

Eight extractions were performed using EDTA-treated normal human plasma pooled from over 100 HIV-negative individuals. A single 1×10^4 IU/mL (5.8×10^3 c/mL) standard was also extracted as a positive process control. After same-day viral load testing, an RNastable

preparation was made from each process control extraction followed by resuspension and evaluation after storage for 7 days at 45°C.

2.7 Statistical analysis

All viral loads were transformed to a $\log_{10}$ scale. Transformed data was used to calculate the change in detectable viral load between treatments and time points. Average changes in viral load and the associated standard deviation were then calculated for each treatment group. Samples with values below the assay LOD of 500 c/mL were excluded from these calculations.

Logarithmically transformed data from different treatment groups were plotted against one another, and the associated R^2 was calculated. If the viral load for a sample was undetectable or below the assay cutoff for either of the time points in a given comparison, the sample was not included in the linear regression or calculations of change in viral load. Bland-Altman plots were used to visualize the agreement of the viral load determinations from different days and sample treatments (Bland and Altman, 1995). Data sets (two time points) with one or more time-point readings below the assay cutoff were not graphed, since they would introduce an artificial bias due to the $2.7 \log_{10}$ c/mL cutoff value.

3. Results

3.1 Post-stabilization real-time amplification

For this study, viral load was determined using a modified G2 LTR RT-PCR protocol (Rouet, et al., 2005; Rouet, et al., 2010). In the standard protocol using 20- μ L template in 50- μ L total PCR volume, significant signal quenching was observed. At a 1:5 dilution of RNA template recovered from RNAstable into the reaction mixture, quenching was no longer observed. For this reason, the protocol was modified to use 20- μ L RNA template in 100- μ L total PCR volume. Under these conditions no impact was observed on the performance of the G2 LTR RT-PCR assay with standard samples. RT-PCR amplification plots for cDNA preparations were not directly comparable to the standard curves such that the thresholds were manually selected above the amplification plots of all negative template controls while achieving acceptable linearity for standard curves ($R^2 > 0.950$).

For the data presented below comparison of independent extractions on identical samples performed on separate days produced R^2 and slope values of 0.888 and 1.063, respectively.

3.2 Stabilization of viral RNA through RNAstable

The lowest viral load for which viral RNA was recovered in all stabilization conditions was 2,175 c/mL, which corresponds to stabilization of 145 viral RNA copies per stabilization reaction. These numbers assume 100% extraction efficiency. The only exception was one sample in the RNAstable/Day1 treatment group, which tested negative despite a viral load determination of $4.00 \log_{10}$ c/mL for all other preparations. When compared with baseline viral load values, all time points evaluating RNAstable preparations produced strong linear correlation with R^2 values ranging from 0.871 to 0.943 with slopes ranging from 0.941 to 0.987 (Figure 2). For all correlations $p < 0.0001$.

Specimen stabilization by RNAstable resulted in an average drop in viral load of less than 0.52 (average ± 1 standard deviation) $\log_{10}$ c/mL (Table 1). This drop in viral load probably results from the process of stabilization and resuspension of the viral RNA from the RNAstable, since very little average change in viral load was observed between RNAstable preparations (Table 2).

Bland-Altman plots for all comparisons to baseline viral loads showed that the correlations are not biased by variation in HIV-1 RNA concentrations (Figure 3). Some data was excluded from change in viral load calculations due to viral loads that were undetectable or below the cutoff value for one or both of the preparations within a given comparison (see Table 1). The highest viral load detected for any of these excluded samples was 1,905 c/mL ($3.28 \log_{10}$ c/mL).

3.3 Stabilization of viral RNA signal through reverse-transcription

For specimens stored in liquid as cDNA, the lowest viral load for which signal was recovered was 2,175 c/mL, which corresponds to stabilization of 145 viral RNA copies per stabilization reaction. cDNA preparations yielded a strong correlation ($R^2=0.762$, slope=1.173) when compared to baseline viral loads (Figure 2) with $p<0.0001$. The average change in viral load between specimens was $-0.01 \pm 0.367 \log_{10}$ c/mL (Table 1). There was no substantial change in the mean viral load of all samples when comparing any preparation types (Table 3).

The Bland-Altman plot comparing real-time PCR Day7 data to baseline viral loads showed that the correlation is not biased by variation in HIV-1 RNA concentrations (Figure 3). The highest viral load detected for any of these excluded samples was 1,905 c/mL ($3.28 \log_{10}$ c/mL).

3.4 Standard curves and controls

All negative controls, extractions of normal human plasma, and negative processing controls tested negative. All positive processing controls had detectable viral loads determined to be within $0.50 \log_{10}$ c/mL of the expected value (data not shown).

4. Discussion

This study demonstrates two methods that allow for the delayed quantification of extracted HIV-1 RNA after long-term storage at room temperature. One approach uses a commercially available product, RNastable, in which the viral RNA is stored as a component of a dry matrix. The other approach simply involves reverse transcription of the RNA and stabilization of the cDNA in liquid format.

A previous study demonstrated the stability of RNA in RNastable, qualitatively for three specimens over 11 days at room temperature (Wan et al., 2009). This study demonstrates the ability of RNastable to support purified RNA from 145 HIV genome copies (2,175 c/mL) reliably for 92 days at 45°C. Furthermore, the viral RNA dry-down and recovery process has no substantial effect on quantification across the dynamic range of 500 to 1.6×10^6 HIV genome copies/mL. The data suggests that despite an apparent drop in viral load due to the RNastable dry-down and/or resuspension process, the sample is highly stable when dry. DBS and DPS which stabilize viral RNA prior to RNA purification result in similar limits of detection between 1000 and 10,000 c/mL, with perhaps slightly lower quantitative correlations below 10,000 HIV genome c/mL. A limitation of the RNastable product was an incipient in the product formulation that impacted the real-time analysis of the PCR possibly due to quenching of the fluorescent signal. This meant that the PCR had to be performed at a 1:5 dilution of template (20 μ L in 100 μ L total reaction volume) rather than the current G2 LTR RT-PCR reaction conditions of 1:2.5 dilution (10 μ L in 25 μ L total reaction volume). At a 1:5 dilution of template there was no observed impact of the incipient on the PCR itself. The data suggests that this class of products will be very useful for storage at room temperature and shipment of viral RNA extracted from clinical specimens in the context of specimen repositories and multicenter studies (Hernandez et al., 2009, Wan et al., 2009,

Frippiat et al., 2011). Further work needs to be done to validate this on other viruses and other specimen sources. Modifying RNASTable or developing a similar product to include an internal process control, allow for smaller PCR volumes, and eliminate the need for a vacuum concentrator to dry down low concentrations of RNA efficiently would make this an attractive modular tool to support point-of-care extraction methods (Puren, et al., 2010). Genvault-IntegenX has developed a device to standardize dry down protocols for their GenTegra products. Additional work is required with the current product to establish tolerance values for sample dry-down and rehydration times and to evaluate the interpreparation variation.

Viral RNA signal was also stabilized in liquid format through sequence-specific reverse transcription of RNA extracted from clinical specimens. The resulting cDNA in the reverse-transcription reaction mixture was remarkably stable, and was reliably detected at the equivalent of 145 viral genome copies (2,175 c/mL) after 7 days at 45°C. It should be noted, however, that the amplification plots for cDNA preparations were not directly comparable to standard curves or RNASTable preparations and further optimization would have to be performed to evaluate this format accurately. Even with these limitations, the correlation was still strong when comparing baseline viral loads to cDNA preparations after conversion to a ranked scale ($R^2=0.779$, slope=0.883). A limited number of specimens that were stored for 92 days at 45°C did not return any results (data not shown), more detailed stability studies are required to understand the kinetics of decay in this specimen type.

RNA lability imposes additional demands on systems supporting specimen handling, storage, and shipment for molecular diagnosis of RNA viruses. Stabilization methods such as dried filter spots and ViveST™ are extremely valuable for collection and handling of clinical specimens. There are, however, very few options for handling extracted viral RNA beyond storage in chaotrope solutions and under cold chain. Stabilization methods in this domain will provide flexibility in laboratory systems supporting molecular diagnosis of RNA viruses and may play a critical role in supporting emerging nucleic acid amplification platforms designed for use in more decentralized and resource-limited settings. This study demonstrates that a new generation of specimen storage products such as the RNASTable products warrant further investigation for their suitability in viral pathogen detection applications.

Highlights

- Two approaches to quantitatively stabilize HIV RNA were evaluated
- RNASTable, a commercial product, stabilized 145 genome copies at 45°C for 92 days
- Viral load could be accurately determined from RNA recovered from this product
- Viral load could be determined from cDNA stored at 45°C for 7 days

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Abbreviations

ART	anti-retroviral therapy
DBS	dried blood spots
DPS	dried plasma spots
G2 LTR RT-PCR	Real-time polymerase chain reaction based viral load detecting the long terminal repeat domain of HIV
LOD	limit of detection
RT-PCR	Real-time polymerase chain reaction

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Figure 1. Stability Study Timeline

Diagram of sample processing scheme described in sections 2.5.1, 2.5.2, and 2.5.3. Baseline, cDNA Day 7, and Day 1 and Day 7 RNAsable RT-PCR values were determined from samples prepared from a pooled duplicate extraction for each repository clinical specimen. Day 92 RNAsable was prepared from a separate RNA extraction.

Figure 2. Correlation of Viral Loads for RNAstable Resuspensions Versus Day 0 Plasma Extractions

All comparisons represent data with viral loads >500c/mL at both time points. Undetectable viral loads were plotted using a value of 500 c/mL for visualization purposes only and were not included in calculations or analysis. This data is graphed as empty circles. Dashed lines indicate the 500c/mL cutoff. Viral load determinations are plotted for: a. RNAstable resuspensions at Day 1, b. Day 7, c. Day 92, and d. cDNA at Day 7. These are plotted against baseline viral load determinations from extracted plasma at Day 0.

Figure 3. Bland-Altman Comparisons of HIV-1 Viral Load Determinations

The following preparation comparisons are shown: a. Resuspension of RNAstable dry-down at Day 1 versus extraction from freshly thawed plasma at Day 0, b. Resuspension of RNAstable dry-down at Day 7 versus extraction from freshly thawed plasma at Day 0, c. Resuspension of RNAstable dry-down at Day 92 versus extraction from freshly thawed plasma at Day 0, d. Evaluation of cDNA preparations at Day 7 versus extraction from freshly thawed plasma at Day 0, e. Extraction from freshly thawed plasma at Day 7 versus extraction from freshly thawed plasma at Day 0.

Change in Viral Load Determinations

Table 1

Mean change in viral load log₁₀ (copies/mL) when comparing individual samples at retesting, and baseline viral loads at Day 0. Standard deviations are provided in brackets. Change in viral load is shown for specimens stored for 1 day, 7 days, and 92 days in RNAstable, and 7 days as cDNA. In addition, change in viral load between plasma extraction at day 0 and extraction from the same plasma stored at -80°C and thawed at Day 7 are shown. Samples with detectable viral loads at both Day 0 and upon retesting were used in the regression and change in viral load calculations.

Plasma	RNAstable			cDNA		Plasma	
	Day 1	Day 7	Day 92	Day 7	Day 7	Day 7	Day 7
Day 0							
							</

Table 2
Comparisons of Change in Viral Load Determinations Within Stabilized Specimens and Plasma Taken at Day 1 and Day 7

Data is shown for average changes in viral load $\log_{10}$ (copies/mL) among individual specimens at Day 1, Day 7, and Day 92 in RNAstable. Standard deviations are provided in brackets. Samples with detectable viral loads at both testing times were used in the regression and change in viral load calculations.

		RNAstable	
		Day 7	Day 92
RNAstable			
Day 1			
	<i>Mean change of viral load</i>	-0.04	-0.06
	<i>Standard Deviation</i>	± 0.284	± 0.204
	<i>Samples with detectable viral loads at both Day 1 and upon retesting</i>	32	16
Day 7			
	<i>Mean change of viral load</i>		-0.14
	<i>Standard Deviation</i>		(± 0.213)
	<i>Samples with detectable viral loads at both Day 7 and Day 92</i>		15

Mean Viral Load Determinations

Table 3

Average viral loads $\log_{10}$ (copies/mL) and standard deviations were calculated within a given preparation and time point group for all samples with viral loads >500 copies/mL. Standard deviations are provided in brackets. *n* indicates the total number of samples within the treatment group with a viral load greater than 500 copies/mL. One RNAstable treated sample had a detectable viral load at Day 1 (759 copies/mL), but was previously undetectable at Day 0. This was also the case for one sample in the cDNA treatment group (1549 copies/mL), and three samples retested from freshly thawed plasma at Day 7 (813, 851, and 1288 copies/mL).

	Plasma		RNAstable		cDNA	
	Day 0	Day 7	Day 1	Day 7	Day 92	Day 7
Average viral load	4.05	3.92	3.94	3.94	3.80	3.97
Standard deviation	(± 0.782)	(± 0.779)	(± 0.785)	(± 0.752)	(± 0.782)	(± 0.730)
n	45	33	38	37	21	24