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Efficient Methodologies for Sensitive HIV-1 RNA Quantitation from Plasma and Vaginal Secretions

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Abstract

Background—Quantifying HIV levels in mucosal secretions is essential to study compartmentalized expression of HIV and facilitate development of intervention strategies to prevent disease progression and transmission.

Objectives—To develop a sensitive, reliable, and cost-effective technique to quantify HIV from blood and vaginal secretions that is compatible with efficient implementation in clinical research environments.

Study Design—A sensitive, reliable, internally-controlled real-time reverse transcriptase (RT) PCR assay, which uses the HIV-1 *pol* gene as a target (Hpol assay) was developed to quantify HIV levels in plasma and genital secretions, and compared to the widely-used Roche Amplicor HIV-1 Monitor assay. In addition, a simplified method of sample collection and processing of genital secretions (self-collection and use of *RNAlater* with batch processing) was compared to provider collection of samples and immediate processing.

Results—The sensitivity and reliability of HIV levels detected by the assay described herein correlate well with measurements from Roche Amplicor™ HIV-1 Monitor assay for both plasma and vaginal secretions ($R^2=0.9179$ and $R^2=0.942$, respectively). The Hpol assay reproducibly quantifies a lower limit of 5 HIV-1 RNA copies per reaction, with low-levels of inter-assay and intra-assay variation. Additionally, vaginal viral levels and detection frequency did not differ significantly between the two the collection and processing methods.

Conclusions—The methodologies developed here provide sensitive, reliable, and cost-effective quantification of HIV levels in plasma and mucosal secretions, and are compatible with efficient use in clinical research studies.

Keywords

Human immunodeficiency virus; real-time PCR; genital; plasma; *RNAlater*

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1. Background

Quantifying HIV levels in mucosal secretions is critical to understand viral expression mechanisms and develop suitable intervention strategies for disease progression and transmission⁽¹⁻⁵⁾. In clinical research environments, cost and efficiency of analyses favor the use of in-house real-time PCR quantitation assays over commercial assays. Several such assays have been described, but few have been validated to monitor HIV expression in mucosal secretions or have included an internal control to monitor efficiency of RNA recovery, reverse transcription (RT), and inhibition of PCR amplification⁽⁶⁻⁹⁾. Samples collected from the female genital mucosa are frequently collected during a pelvic examination. To preserve viral RNA integrity, these samples are typically held on ice and processed immediately. This process can be cumbersome and inefficient, especially for large clinical studies.

2. Objectives

We sought to streamline methodologies used to collect genital secretions and evaluate HIV levels in clinical research studies. The objectives of this study were: (1) to develop a cost-effective, internally-controlled, reproducible, and sensitive real-time RT PCR assay to quantify HIV-1, (2) compare viral level analyses of self-collected vaginal secretions with samples collected by providers via pelvic exam, and (3) evaluate the use of *RNAlater* preservative solution for sample storage and weekly batch processing.

3. Study Design

3.1. Specimen Collection and Processing

Informed consent for sample collection was obtained in accordance with institutional review board of Louisiana State University Health Sciences Center. Peripheral blood and vaginal secretions were collected from women (>18 years) attending an HIV outpatient clinic in New Orleans, LA. HIV-negative subjects were recruited for control samples.

EDTA plasma samples were stored at -80°C in 1mL aliquots. Vaginal secretions were collected using polyester-tipped swabs (Fisher catalog #23-400-111). Before pelvic examination, randomly selected subjects were instructed to self-collect genital secretions and deposit samples in an empty sterile collection tube (15mL conical polypropylene tube, used throughout study). Afterwards, provider-collected samples were obtained from the vaginal vault during pelvic examination and also placed in empty collection tubes. Samples were always collected in the order described.

For immediate processing, vaginal secretion swabs (self and provider collected) were placed in collection and transported to the laboratory on ice. Secretions were eluted in 1mL PBS by vortexing 30 seconds, fractioned by centrifugation ($1,500 \times g$ for 10 minutes) and supernatants were stored at -80°C within 3 hours of collection. For batch-processing, provider-collected swabs were immediately placed in collection tubes containing 1 mL of *RNAlater* (Ambion) preservative solution and stored at 4°C for up to 2 weeks. On a weekly basis, vaginal samples stored in *RNAlater* were eluted from the swab and fractioned as described above and stored at -80°C .

3.2. HIV-1 Quantitation Assay

The HIV *pol* region was selected as the target region for PCR amplification due to its highly conserved nature among clade B HIV genomes⁽¹⁰⁾. To generate a quantitation standard, a 664 base pair region spanning the HIV-1 *pol* gene was PCR-amplified from HIV_{HXB2} (nucleotides 2597 to 3261) and cloned into the pCR 2.1 TOPO vector (Invitrogen, CA). The

resultant plasmid, pHXB-pol, was serially diluted 10-fold and used as the DNA standard for HIV quantitation. DNA was purified from the cell line ACH-2 (NIH AIDS Research and Reference Reagent Program) to verify quantitation assay accuracy. This cell line contains one copy of HIV proviral DNA per cell. To prepare an HIV RNA template for use as a quantitation standard, a 794 bp transcript was produced from pHXB-pol by T7 RNA polymerase (RiboMax *In vitro* Transcription System, Promega). The resultant RNA was purified, quantified by spectrophotometry, and serially diluted to contain 10^7 – 10^1 copies of the *pol* RNA template. DNA template removal was confirmed by real-time PCR under RT-excluded conditions.

3.3. Exogenous RNA Internal Control

The exogenous internal control RNA was derived from plasmid pMKCP3, containing sequences from brome mosaic virus (BMV) RNA 3, generously provided by Dr. Paul Ahlquist⁽¹¹⁾. A 2273 base RNA template, IC_{BMV}, was produced by *in vitro* transcription from pMKCP3 using the RiboMax system (Promega, WI). 300 pg/mL IC_{BMV} RNA was added to each sample in Trizol reagent (Invitrogen) as described below. IC_{BMV} RNA and virion RNA were simultaneously purified and detected in a multiplexed real-time RT PCR assay, utilizing IC_{BMV}-specific and HIV*pol*-specific primers and probes (Table 1).

3.4. HIV- Hpol Real-time RT PCR Multiplex Assay

Virions were pelleted from 1 mL vaginal secretion or plasma aliquots ($20,000 \times g$ for 1 hour at 4°C), supernatants were removed, 1mL Trizol reagent containing 300 pg/mL IC_{BMV} was added, and RNA was purified per the manufacturer's protocol. Ten-fold serial dilutions of the Hpol RNA template (diluted in RNase-free water containing 100µg/mL glycogen) were included in each assay to provide a standard curve for quantitation. The Hpol standards and one-tenth of total RNA from samples were reverse-transcribed in a reaction containing TaqMan PCR Buffer II, 5.0 mM MgCl₂, 2.5 µM random hexamers, 25 units MultiScribe reverse transcriptase and incubated for 10 minutes at 25°C, 30 minutes at 48°C, 5 minutes at 95°C, and 5 minutes at 4°C. The PCR master-mix was added to the resultant cDNA by overlay (1X TaqMan Buffer A, 5.5 mM MgCl₂, 0.5mM dNTP, 1.25 units AmpliTaq Gold, 450nM of both HpolF and HpolR, and 250nM HpolProbe). When multiplexed, the reaction mixture also contained 125 nM IC-F and IC-R and 70nM IC-Probe.

Amplification was performed using an ABI 7300 through the following: 10 minutes at 95°C prior to 40-45 cycles of 94°C for 15 seconds and 60°C for 1 minute. HIV copy number was determined by plotting the threshold cycle (C_T) value of the unknown on the standard curve derived from the Hpol RNA standards. Inhibition was inferred if the C_T value for IC_{BMV} was >2.5 units from the reference C_T . Two HIV-negative plasma samples, one HIV-positive sample (described below) and two IC_{BMV} only reference samples were included in each assay. HIV levels were reported as copies per mL (plasma) or swab (secretion supernatant). For Roche Amplicor™ HIV-1 Monitor Assay quantitation, viral RNA was purified and quantified using the “ultra-sensitive” method (COBAS, version 1.5), per the manufacturer's recommended protocol⁽¹²⁾.

3.5. HIV-Positive Control Samples

HIV-positive control samples were prepared using a virus stock (HIV_{JRFL}) obtained from supernatants of infected cultures. Frozen aliquots of the stock contained 2.0×10^9 copies/mL as determined by the Hpol. For validation experiments, HIV-negative plasma and vaginal secretion samples were spiked with serial dilutions of HIV_{JRFL} culture aliquots and stored at –80° C. HIV-positive control samples for quantitation assays were prepared by spiking 1mL normal plasma with 5×10^4 copies of HIV_{JR-FL}. For experiments to validate IC_{BMV}

inclusion, HIV-negative plasma and vaginal secretion samples were spiked with serial dilutions of HIV_{JRFL} stock aliquots prior to storage at -80°C .

3.6. Statistical Analyses

Linear regression analysis, Yates-corrected chi-square (X^2), Fisher's exact test, and Mann-Whitney Wilcoxon sum measures of statistical significance were used for viral level comparisons. For statistical analysis, samples with undetectable levels of HIV (<50 copies) were assigned the value of 35.4 copies/ml or swab ($L/2$, where L =limit of detection, 50 copies)⁽¹³⁾. Viral copy numbers were $\log_{10}$ transformed for analyses.

4. Results

4.1. Validation of TR-Hpol Real-time RT PCR Assay

The quantitation assay was first validated for DNA amplification. Consistently reproducible measures of sensitivity and accuracy were observed when serial 10-fold dilutions of pHXB-pol were amplified. Assay accuracy was verified by amplifying proviral DNA from ACH-2 cells, wherein there is a single HIV-1 integrated provirus. The measured versus expected HIV provirus copy number correlated well ($R^2=0.9973$) in ACH-2 DNA samples. For the RNA standard curve, intra- and inter-assay comparisons validated assay reproducibility, with only nominal variation observed (Table 2). Comparisons of observed assay variations with previously published assays indicate that the methods described here meet requirements for reproducible and accurate quantitation^(9, 14).

To further validate the Hpol assay, HIV levels from 36 plasma and 20 vaginal samples were quantified by the Roche Amplicor HIV-1 Monitor Assay⁽¹⁵⁾ alongside the Hpol real-time RT PCR assay. Matched HIV RNA levels from each assay were highly similar for plasma and vaginal HIV levels (Figures 1A and 1B). In addition, there was no significant difference in the frequency of detection with either method or sample type. Reagents utilized in the real-time assay are commercially available from several sources, resulting in a significant reduction in reagent cost as compared to the commercial quantitation kits.

To evaluate the sensitivity of the Hpol assay, aliquots HIV_{JR-FL} culture supernatant (2.0×10^9 copies/mL as determined by independent quantitation) were serially diluted to contain an estimated 100, 50, 30, and 10 copies/mL and spiked into HIV-1 negative samples. Because 1/10 of total RNA is evaluated in each reaction, this test evaluated the ability of the Hpol assay to detect 1, 3, 5, and 10 copies of HIV-1 RNA per reaction. The results of this analysis (Table 3) indicate the limit of accurate quantitation using the Hpol assay is 5 copies of HIV-1 RNA per reaction, translating to 50 copies per mL/sample. By evaluating sample RNA in duplicate, the probability of not detecting 5 viral copies in at least one replicate is < 0.1%.

4.2. Validation of the Internal Control (IC_{BMV})

To confirm that addition of IC_{BMV} RNA did not interfere with the sensitivity and accuracy of HIV quantification, serial 10-fold dilutions of HIV_{JRFL} (to a limiting dilution) were spiked into HIV-negative plasma and vaginal secretion supernatant samples. For both types of samples, HIV levels quantified in the presence and absence of IC_{BMV} RNA were highly similar ($R^2=0.8693$ and $R^2=0.8154$ for plasma and vaginal secretions, respectively). No significant difference in detection of HIV-1 RNA was observed between the IC_{BMV} inclusive and exclusive groups for both plasma and vaginal secretions as well ($p=0.66$ and $p=0.93$, plasma and vaginal secretions, respectively, Mann-Whitney Wilcoxon rank-sum). At limiting dilutions (estimated at 1-10 copies) of HIV_{JRFL} per sample, detection sensitivity was also not significantly different between the two groups in both sample types.

4.3. Validation of Vaginal Secretion Collection and Processing Protocols

To compare sample collection protocols, vaginal secretions were obtained from 54 subjects by self-collection and a medical provider via pelvic examination. Additionally, immediate processing of samples was compared with a protocol utilizing *RNAlater* preservative solution and weekly batch processing (n=43 subjects). HIV detection frequencies in both the provider vs. self-swab and immediate processing vs. *RNAlater* solution comparisons were not significantly different in either comparison (Table 4). Samples stored in *RNAlater* were not negatively impacted by the preservative solution, as four additional positive samples were detected in this sample set that were undetected in the immediate processing group. Similarly, a marginally higher frequency of HIV detection was observed in self-collected samples compared to provider-collected. HIV RNA was detected in vaginal samples from 15 subjects. HIV was detected in seven samples by both collection protocols, while self-collection identified an additional six positive samples not found in provider-collected swabs. Provider collected samples identified two HIV-positive specimens that were not detected in self-collected samples.

Levels of vaginal HIV were compared between provider or self-swab collection and *RNAlater* or immediate processing, and in both sets of analyses, levels obtained were not significantly different (Table 5). Direct comparisons by linear regression yielded correlation coefficients of 0.78 for both collection and processing methods (Figure 2). Although these correlation coefficients are lower than that observed in comparison of the Hpol assay with the Roche Amplicor assay, it is emphasized that this difference reflects the inherent variability associated with duplicate, independently-collected and processed samples from the same subject. These R^2 values, with which approximately 80% of variation within each comparison is explained by the linear relationship, indicate the validity of self-swab and batch processing methods as compared to provider collection and immediate processing.

5. Discussion

We have described the development and use of an internally-controlled, reliable, efficient, and sensitive methodology to quantify HIV-1 RNA in plasma and vaginal secretions. The assay described here represents a significant improvement over previously described real-time RT-PCR quantitation assays for HIV (8, 14, 16-20), as the inclusion of an internal RNA standard allows monitoring of RNA extraction efficiency, reverse transcription, and inhibition of PCR amplification. Additionally, vaginal viral levels obtained with the described Hpol assay are statistically indistinguishable from those obtained with the Roche Amplicor Monitor assay. The Hpol assay can be used to quantify HIV-1 RNA in other mucosal secretions; its use to quantify HIV-1 levels in cervical secretions has been validated (data not shown) and pilot studies have been conducted in saliva. A commercially-available mixture of BMV RNAs has previously been used as an internal control for HIV-2 quantitation⁽²¹⁾. We have significantly improved this methodology by transcribing a molecular clone of a BMV RNA segment *in vitro*, eliminating variability in the commercial RNA mixture and providing a standard RNA template. Finally, our results validate the use of self-collection of genital secretions and an *RNAlater* batch processing protocol. This protocol facilitates sampling in the absence of a pelvic examination, allows sampling at clinic and non-clinic sites, and permits sample collection independent of laboratory scheduling and immediate processing. These methods also represent a significant reduction in cost due to the use of widely available reagents and reduced personnel time required for batch processing. The combined methodologies provide significantly improved tools with which HIV-1 expression can be comprehensively examined in multiple compartments with accuracy and sensitivity. They will permit more frequent sampling, and thereby augment

longitudinal clinical research studies in settings where the cost of commercially available assays is prohibitive and laboratory facilities are limited.

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Abbreviations

HIV/HIV-1	human immunodeficiency virus/ human immunodeficiency virus type 1
PCR	polymerase chain reaction
RT	reverse transcriptase
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
PBS	phosphate buffered solution
BMV	brome mosaic virus

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Figure 1. Comparison of Plasma and Vaginal HIV Levels Derived from the Roche Amplicor System and the Hpol Real-time RT PCR Assay

Comparisons were conducted from duplicate aliquots of a single 1 ml sample of plasma and provider-collected vaginal secretion supernatants; 500 ul of each sample were analyzed in the tandem assays. **A.** Comparison of Log_{10} value from plasma samples ($n=36$). HIV RNA was detected in 30 plasma samples using the real-time RT PCR assay and 31 samples using the Roche Amplicor system. **B.** Comparison of Log_{10} HIV levels from vaginal secretion supernatant samples ($n=20$). HIV RNA was detected in all vaginal secretion supernatant samples analyzed.

Figure 2. Comparison of Collection and Processing Methodologies

A. Self-collected vs. Provider Collected Vaginal secretion samples. Comparisons of HIV levels detected from two independent vaginal swabs collected from the same participant (n=54), within a 30 minute time span, with immediate processing for storage. B. RNAlater vs. Immediate Processing Methodologies. Comparisons of HIV levels detected from duplicate samples collected simultaneously from subjects (n=43) by a provider during a pelvic exam and processed by different methodologies as described. .

Table 1

Primers and Probes used in Taq-Man Assays

Primer and Probe Sequences	
HpolF	GAAATTTGTACAGAAATGAAAAAGGAA
HpolR	TGAGTTCTCTTATTAAGTTCTCTGAAATCTAC
HpolProbe	6FAM—TTGGGCCTGAAATCCATACAATACTCCAGT—TAMRA
IC-F	GCTCGCAGAAATCGTTGGA
IC-R	CAGCGAGTGGTTCGACAATTAC
IC-Probe	VIC—CCGCTAGGGTCCAAC—TAMRA

Primer and probe sets were designed using *Primer Express* software, (version 1.0, Applied Biosystems, Foster City, CA), and obtained via custom order from Applied Biosystems.

Table 2
Intra-Assay and Inter-Assay Variation in Amplification of Hpol Standard in 5 Experiments

Standard Copy Number	C_T Value Exp. 1	% CV Exp.1	C_T Value Exp. 2	% CV Exp.2	C_T Value Exp. 3	% CV Exp.3	C_T Value Exp. 4	% CV Exp.4	C_T Value Exp. 5	% CV Exp.5	% Intra-Assay Variation
10	37.2 ± 0.02	0.5	38.1 ± 0.05	0.1	37.9 ± 0.27	0.7	38.1 ± 0.22	0.6	38.3 ± 0.16	0.4	1.1
100	33.9 ± 0.06	0.2	34.6 ± 0.19	0.6	34.5 ± 0.07	0.2	34.8 ± 0.22	0.6	34.9 ± 0.07	0.2	1.5
1,000	30.6 ± 0.31	1.0	31.3 ± 0.05	0.2	30.0 ± 0.12	0.4	31.3 ± 0.08	0.2	31.5 ± 0.27	0.8	1.4
10,000	27.7 ± 0.29	1.0	28.2 ± 0.52	2.0	27.1 ± 0.03	0.1	27.6 ± 0.14	0.5	27.9 ± 0.06	0.2	1.8
100,000	25.1 ± 0.03	0.1	24.7 ± 0.01	0.2	23.6 ± 0.12	0.5	24.1 ± 0.08	0.3	24.4 ± 0.18	0.7	2.1
1,000,000	21.5 ± 0.57	3.0	21.1 ± 0.00	0	20.1 ± 0.08	0.3	20.4 ± 0.08	0.4	20.9 ± 0.21	1.0	2.5
10,000,000	17.7 ± 0.29	2.0	17.9 ± 0.09	0.5	16.7 ± 0.03	0.2	17.0 ± 0.11	0.6	17.5 ± 0.11	0.6	2.2
Assay Values	Slope: -3.31 Y-intercept: 40.12 Corr. Coeff: 0.991		Slope: -3.30 Y-intercept: 40.95 Corr. Coeff: 0.997		Slope: -3.36 Y-intercept: 39.26 Corr. Coeff: 0.991		Slope: -3.39 Y-intercept: 40.04 Corr. Coeff: 0.996		Slope: -3.38 Y-intercept: 39.11 Corr. Coeff: 0.998		

C_T Value – mean threshold cycle at which respective standard copy number is detected ± standard deviation; %CV – inter-assay percent coefficient of variation between replicates; Corr. Coeff – correlation coefficient (degree to which individual standard copy number data points fit the slope of the standard curve, exact fit = 1.00); C_T values representative of triplicate analyses from 5 independent standard curves.

Table 3

Sensitivity of Detection with Hpol Quantitation Assay

Input Amount	% Detection
1 copy	56% *
3 copies	73%
5 copies	97%
10 copies	100%

Hpol RNA templates were diluted to the estimated copy number and detection frequencies determined from analysis of 6 replicates at each dilution in 5 independent quantitation assays (total of 30 replicates per dilution).

* 55.6% detection fits expected Poisson probability of one copy detection rate ⁽²²⁾; observed versus expected rates of detection were not statistically different (Yates-corrected χ^2 1.461, $p > 0.20$)

Table 4

Detection Frequencies in Methodology Comparisons

Methodology	% Detection Vaginal HIV	<i>p</i> Value [†]
Self-Swab Collection	24.2% (13/54)	0.47
Provider Collection	16.7% (9/54)	
RNAlater Solution	26.8% (12/43)	0.44
Immediate Processing	19.5% (8/43)	

[†] Yates-corrected chi-square test

Table 5

Comparisons of Viral Levels Obtained with Methods of Processing and Collection

Methodology	Mean Vaginal Viral Level (copies/swab)	Range Vaginal Viral Level (copies/ swab)	Comparison *
<i>RNAlater</i> processing ⁺	651.4	<50 – 19,952	$p=0.52$
Immediate processing	884.4	<50 – 16,595	
Self-swab collection [#]	4840.7	<50 – 222,387	$p=0.53$
Provider collection	3896.0	<50 – 191,953	

Median vaginal HIV RNA value for all methodologies was 50 copies per swab.

⁺ comparison of duplicate samples collected by a provider from 43 subjects

[#] comparison of samples collected from 54 subjects; self-collected samples were collected approximately 30 minutes prior to provider-collected specimens

* Mann Whitney Wilcoxon Rank Sum; Variability in levels is expected due to independent samplings and virus quantitation near the level of detection.