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Quantifying HIV-1 Persistence with Next Generation HIV-1 RNA Single Copy Assays

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Abstract

Background: A qPCR assay with single copy sensitivity targeting HIV *gag* (gSCA) has been widely used to quantify persistence of low-level plasma viremia in the context of antiretroviral therapy (ART). However, mismatches between primers/probe and HIV *gag* sequences result in inefficient amplification in 15-30% of plasma samples with North American subtype B HIV-1. Additionally, recovery of the spiked internal standard (avian sarcoma virions, RCAS) is often <50%, which is suboptimal. To addresses these limitations, we developed a next generation SCA by optimizing purification and targeting integrase (iSCA).

Methods: Primers/probe were designed to target a region identified as highly conserved in *pol*. Recovery of naked HIV RNA transcripts and spiked internal standard was assessed by qPCR to identify an optimal extraction method. Paired plasma samples for comparison of gSCA and iSCA performance were collected from 35 subjects (25 with viremia suppressed on ART for >2 years; 10 with viremia >50 copies/mL). The efficiency of gSCA and iSCA were compared to levels of viremia obtained with the Roche COBAS AmpliPrep/Taqman System (v2.0). Using a novel approach (Mega-iSCA), large volumes of plasma were assayed to further improve the sensitivity of iSCA.

Results: In the test panel of 35 paired plasma samples, median recovery of RCAS was 1.5 times greater with iSCA than gSCA (p<0.001, signed rank test). Of the 10 samples with viremia >50 copies/mL by Roche Taqman, gSCA efficiently amplified HIV-1 RNA from 7 patients, whereas iSCA amplified HIV-1 RNA efficiently from all 10 patients (p=0.038, exact binomial test). For samples from the 25 patients on ART,10 of 25 had greater than 3-fold higher recovery of HIV-1 RNA with iSCA versus gSCA compared to 5 of 25 with gSCA versus iSCA (p<0.021, exact binomial test). Mega-iSCA led to the detection of HIV-1 RNA in 4 of 5 patients who were previously undetectable with iSCA.

Conclusions: This next generation plasma HIV-1 RNA single copy assay (iSCA) improves viral RNA recovery and reduces primers/probe mismatch such that inefficient amplification and false negative results are less frequent than with earlier assay versions. Furthermore, Mega-iSCA improves the sensitivity of the assay to allow quantification of low-level viral load in patients that were previously undetectable.

Introduction

- **The single copy assay (gSCA)[1-4] developed in 2003 has been widely used to quantify low-level viremia**
- **However, nucleic acid extraction is suboptimal and mismatches in primers/probe binding sites are common with gSCA**

Objectives

- **Develop new primers/probe that have more fewer mismatches based on sequences from 2012**
- **Optimize the nucleic acid extraction method to improve HIV-1 RNA recovery**
- **Improve sensitivity by assaying larger plasma volumes**
- **Compare gSCA versus iSCA in a panel of patients**

Materials and Methods

Study Population and Entry Criteria: HIV-1 patients from the Pittsburgh AIDS Center for Treatment (PACT) at the University of Pittsburgh Division of Infectious Diseases were selected as volunteer blood donors. This study was conducted with the approval of the University of Pittsburgh IRB.

Isolation of Nucleic Acids from Plasma: Whole blood was collected in tubes containing EDTA, and was then centrifuged at 500 x g for 15 minutes. The plasma was removed and stored at -80°C in 1.5 mL aliquots. For gSCA, iSCA and Mega-iSCA, a known quantity of an internal standard (replication competent avian sarcoma virus, RCAS) was added to thawed plasma. Plasma was then spun at 1200 x g for 10 minutes to remove debris, followed by centrifugation at 175,000 x g for 30 minutes to pellet virions. For gSCA, the original extraction method was used (proteinase K incubation in water, followed by addition of guanidinium thiocyanate and isopropanol)[1]. For iSCA, an optimized extraction method consisting of sequential proteinase K/guanidinium-HCl treatment followed by addition of guanidinium thiocyanate and isopropanol was used. To compare the gSCA and iSCA extraction methods, the original extraction method was used for iSCA where indicated.

Two-step qRT-PCR quantification of plasma HIV-1 RNA: For both gSCA and iSCA, cDNA was synthesized as previously described[1]. Reactions were performed in triplicate for HIV-1 RNA, duplicate for RCAS (internal standard) for both gSCA and iSCA. Real-time PCR primers/probe for gSCA and iSCA were used, respectively.

Statistical Analysis: Differences in ratios were assessed for statistical significance using an exact binomial test, using the gSCA ratio as the hypothesized ratio. Differences in the RCAS recovery were assessed using a paired T-test. All statistical tests were two-sided with α=0.05.

Results

Figure 1. Nucleotide conservation in HIV-1 subtype B

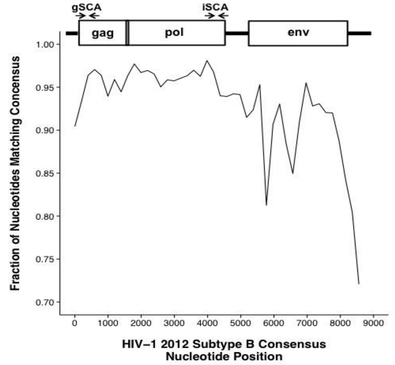
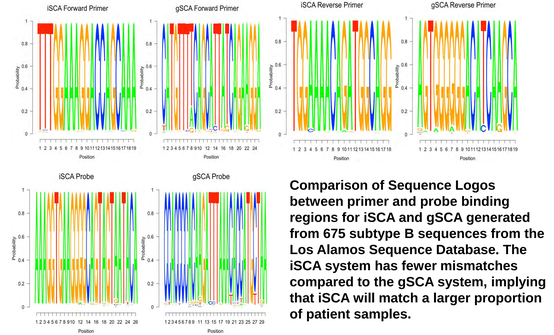


Figure 1. Proportion of nucleotides in 200 base pair fragments that match the HIV-1 2012 subtype B consensus sequence generated from 675 aligned full-length subtype B sequences in the Los Alamos Sequence Database.

Figure 2. Sequence Logos of gSCA and iSCA Primer and Probe Binding Sites



Comparison of Sequence Logos between primer and probe binding regions for iSCA and gSCA generated from 675 subtype B sequences from the Los Alamos Sequence Database. The iSCA system has fewer mismatches compared to the gSCA system, implying that iSCA will match a larger proportion of patient samples.

Table 3. Quantification of plasma viremia with gSCA and iSCA in 10 patients with untreated HIV-1 Infection

Patient ID	Viremia (Log_{10} HIV-1 RNA Copies/mL Plasma)		
	gSCA	iSCA	TaqMan
1	-0.52*§	4.1	4.4
2	3.8	4.3	4.5
3	0.58*	4.3	4.5
4	1.6*	3.2	3.3
5	4.6	4.5	4.9
6	3.0	3.1	3.3
7	5.6	5.7	5.6
8	5.0	5.0	4.8
9	4.6	3.6	3.8
10	2.5	2.9	3.1
Median (IQR)	3.4 (1.9 to 4.6)	4.2 (3.3 to 4.5)	4.2 (3.4 to 4.7)

* Sample with <10-fold recovery compared to Roche TaqMan
§ Sample below the limit of detection of the assay

Recovery of HIV-1 RNA was within 10-fold of the TaqMan platform in 7/10 samples with the original gSCA compared to 10/10 samples with iSCA (p=0.038, exact binomial test comparing iSCA to gSCA).

Figure 3. Quantification of plasma viremia with gSCA and iSCA in 25 patients on ART

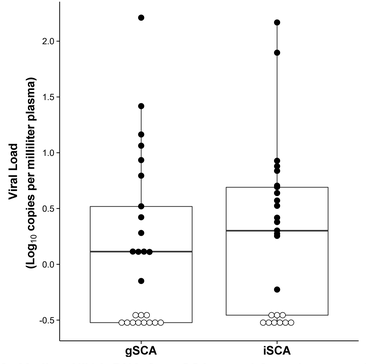


Figure 3. Median HIV-1 RNA for gSCA was 1.3 copies per milliliter of plasma (range: <0.3 to 160) compared with 2.0 copies per milliliter for iSCA (range: <0.3 to 147). HIV-1 RNA values with iSCA were >3-fold gSCA in 10/25 patients, whereas gSCA values were >3-fold than iSCA in 5/25 (p=0.021, exact binomial test).

Table 1. Improved recovery of patient-derived HIV-1 RNA using a new extraction method

Sample	Extraction Method	Number of Replicates	Expected HIV-1 RNA (copies per milliliter)	Mean HIV-1 RNA detected (copies per milliliter)	Mean Percent Expected HIV-1 RNA detected
Viremic HIV-1 Patient Plasma 1:100 Dilution	1.0	4	271	98.3	36%
	2.0	4		292	108%
Viremic HIV-1 Patient Plasma 1:1000 Dilution	1.0	6	27.1	11.6	43%
	2.0	6		23.2	86%

Table 2. Improved nucleic acid recovery in Virology Quality Assurance (VQA) samples with a new extraction method

Assay	Sample	Extraction Method	Number of Replicates	HIV-1 RNA Recovery (copies per mL plasma)	RCAS (Cycle Threshold)
gSCA	VQA 0	1.0	4	<0.2	24.4
iSCA			12	<0.2	24.4
		2.0	5	<0.2	23.5
gSCA	VQA 5	1.0	5	5.7	24.4
iSCA			9	5.1	24.4
		2.0	8	8.3	23.9

HIV-1 RNA and RCAS recovery were consistently higher with the new extraction method (2.0) compared to the old extraction method (1.0).

Table 4. Mega-iSCA can be used to increase the sensitivity of iSCA

Patient ID	iSCA			Mega-iSCA		
	Plasma Volume Tested	Total Copies Detected	Copies per Milliliter	Plasma Volume Tested	Total Copies Detected	Copies per Milliliter
11	2.9	8	5.1	25.6	19	1.4
14	2.8	0	<0.7	25.5	71	5.1
18	2.7	0	<0.7	28.9	7	0.44
23	2.8	3	2	33.6	11	0.65
6	2.9	0	<0.7	23.4	0	<0.1
26	2.9	0	<0.6	29.0	4	0.25
27	2.7	0	<0.7	29.4	15	0.94

Using larger input volumes of plasma in Mega-iSCA increased the sensitivity of the assay and led to the detection of HIV-1 RNA in 4 of 5 patients with previously undetectable HIV-1 RNA with iSCA.

Conclusions

- **Primers/probe for iSCA target a highly conserved region of HIV-1**
- **An improved extraction method increases nucleic acid recovery**
- **iSCA efficiently amplified HIV-1 RNA from viremic patient plasma in 10/10 versus 7/10 patients with gSCA**
- **iSCA had >3-fold recovery of HIV-1 RNA compared to gSCA in 10/25 patients on ART**
- **Mega-iSCA detected HIV-1 RNA in 4 of 5 patients who were previously undetectable by standard iSCA**
- **These next generation SCAs should prove useful in detecting changes in viremia after novel therapies**

References

1. Palmer et al. *J Clin Microbiol*. 2003 Oct;41(10):4531-4536
2. Maldarelli et al. *PLoS Pathog*. 2007 Apr;3(4):e46.
3. Palmer et al. *PNAS*. 2008 Mar;105(10):3879-3884.
4. Eriksson et al. *PLoS Pathog*. 2013 Feb;9(2):e1003174.

Acknowledgements

The authors thank the study volunteers and site personnel at the University of Pittsburgh. We also thank all members of the Mellors Lab for assistance. The study was supported by the Pitt AIDS Research Training (PART) Program #5 T32 AI065380-08 and SAIC contract (25XS119) funded by the NCI.

