Assessment and Quantification of Cell Associate Unpliced HIV-1 RNA using Reverse Transcriptase Droplet Digital PCR (RT-ddPCR)

Poster # 405

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ABSTRACT

Background: Quantification of cell-associated HIV-1 RNA is critical to understanding how HIV-1 latency is established and maintained in a reservoir of infected cells during antiretroviral therapy. Droplet digital polymerase chain reaction (ddPCR) can detect and precisely quantify the target nucleic acids without the need for a calibration curve. Previous studies demonstrated that ddPCR is generally comparable to traditional real time-PCR methods for the quantification of both total HIV-1 DNA and episomal 2-LTR circles. In this study, we developed a reverse transcriptase (RT) ddPCR assay and compared to real time RT-PCR for detection and quantification of unspliced cell-associated HIV-1 RNA in both HIV-uninfected cell lines spiked with HIV-infected cells and clinical samples from patients on antiretroviral therapy (ART) with very low-level viremia.

Methods: To evaluate the ability of RT-ddPCR to measure cell-associated HIV-1 RNA, serially diluted (2.5 to 10^6 copies/10 μl RNA input) *in vitro* transcribed HIV-1 RNA was quantified by RT-ddPCR and real time RT-PCR. We analyzed 8 replicates of the 100-copy standard to assess the accuracy and reproducibility of RT-ddPCR. Levels of cell-associated HIV-1 RNA were determined in clinical samples from patients on suppressive ART, and from MT2 cells spiked with the latently-infected cell line ACH2.

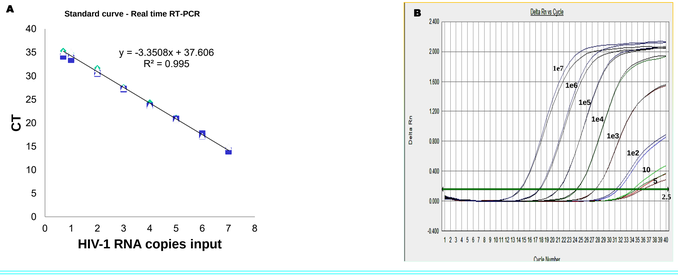
Results: RT-ddPCR accurately quantified HIV-1 RNA transcripts; the dynamic range was 10 to 10^5 copies per 10 μl RNA input (R^2 > 0.99), which represented similar sensitivity as real time RT-PCR. The 100-copy input showed that the percentage of positive droplets most closely correlated with the expected copy number. For samples with input RNA of 10^3 to 10^5 copies, the copies detected was ~10% lower than expected; for samples with <100 copies of input RNA the calculated copy number was slightly higher than expected (mean 1.9-fold difference). Linearity decreased significantly when greater than 10^5 copies due to saturation of positive droplets formation. HIV-1 RNA measured by RT-ddPCR and real time RT-PCR in samples from co-culture of serially diluted reactivated ACH2 cells together with MT2 cells lines were highly correlated (R^2=0.9668). HIV-1 RNA was detected by RT-ddPCR in 14/20 samples (range, 4-284 copies/million PBMC) and by real time RT-PCR in 12/20 samples (range, 6-479 copies/million PBMC), respectively.

Conclusions: The RT-ddPCR assay detected and accurately quantified cell-associated HIV-1 RNA with a dynamic range down to 10 copies per reaction. RT-ddPCR demonstrated similar accuracy and sensitivity compared to real-time RT-PCR in the measurement of cell-associated HIV-1 RNA in both cell-lines spiked with HIV-infected cells and in patient samples. RT-ddPCR is a promising tool for the quantification of HIV-1 cell-associated RNA.

RESULTS

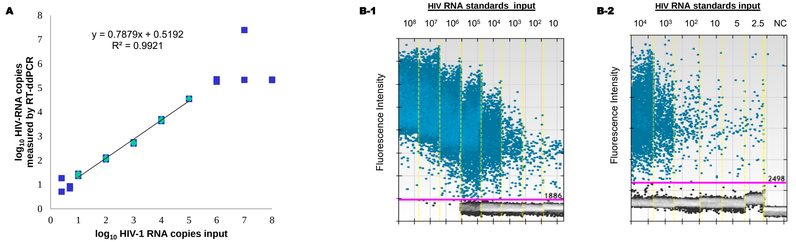
Validation of HIV-1 RNA standards by real time RT-PCR

Figure 1. Standard curve based on serially diluted (10^7,10^6,10^5,10^4,10^3,10^2,10,5,2.5) in vitro transcripts for HIV-1 LTR-gag. The log of the initial template copy number is plotted horizontally, and the cycle number plotted at the crossing point is plotted vertically (panel A). Real time amplification plot in 7300 system-ABI (panel B).



Sensitivity, linear dynamic range and accuracy of RT-ddPCR assay

Figure 2. Standard curve based on serially diluted (from 2.5 to 10^8) in vitro transcripts for HIV-1 LTR-gag. The x-axis is log of the initial template copy number, and the y-axis is duplicates of log of the copy number measured by RT-ddPCR (panel A), the dynamic range was 10 to 10^5 copies per 10 μl RNA input and R2 > 0.99. RNA standards measured by Qx100 Droplet Digital PCR system-BioRad (panel B, B-1 shows detected range from 10 to 10^8; B-2 shows detected range from 0 to 10^4).



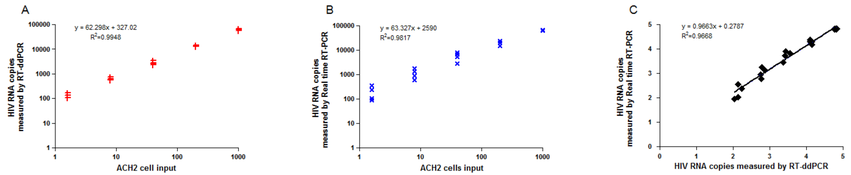
Accuracy & repeatability of RT–ddPCR

Table 1. RT–ddPCR for measurement of 100 copies input of HIV-1 RNA standard with 8 replicates and raw data from RT-ddPCR were 3.0 - 5.2 copies/μl reaction and total reaction volume was 20 μl per well including 8.1 μl RNA input , so the copies/ 10 μl measured by RT-ddPCR were 74.3 – 127.4 copies.

Replicate	RNA input: copies/10 μl	RNA copies/μl measured by RT-ddPCR	copies/10μl RNA input measured by RT-ddPCR
1	100	4.30	106.2
2	100	4.44	109.6
3	100	4.13	102.0
4	100	3.01	74.3
5	100	4.65	114.8
6	100	4.11	101.5
7	100	4.25	104.9
8	100	5.16	127.4
Mean + SD	100 + 0.00	4.3 + 0.61	105.1 + 15.04

Measurement of cell-associated HIV-1 RNA in samples from MT2 **spiked** with latent HIV infected ACH2 cells

Figure 4. Comparison of RT-ddPCR to real time RT-PCR for detecting cell-associated HIV-1 RNA in artificial seeded samples. RT-ddPCR assay (panel A), Real time RT-PCR assay (panel B) and correlation between two methods (panel C).



Detection and quantification of ca-HIV-1 RNA in clinical patients' samples.

Table 2. Detection and quantification of cell-associate unspliced HIV-1 RNA in PBMC cells from ART treated patients with undetectable viral load by the two methods.

Patient-ID	Duration of ART		CD4[a]	VL[b]	HIV-1 ca-RNA detected by RT-ddPCR[c,d]	HIV-1 ca-RNA detected by Real Time RT-PCR[c,d]
	Age					
B004	26	3.5	699	< 50	undetectable	undetectable
B011	26	3.0	696	< 50	18.0	undetectable
M302	59	3.0	1582	< 50	undetectable	undetectable
M305	52	7.5	859	< 50	135.9	116.9
M308	57	6.8	977	< 50	86.6	68.9
M314	25	4.0	1362	< 50	undetectable	undetectable
M316	26	3.0	539	< 50	151.7	108.2
M318	30	6.0	795	< 50	20.5	52.1
M321	66	5.8	89	< 50	undetectable	undetectable
M327	63	4.3	507	< 50	80.9	112.3
M332	27	1.3	726	< 50	undetectable	undetectable
M330	32	3.1	804	< 50	74.1	57.5
M329	34	5.9	306	< 50	5.7	undetectable
M328	26	2.5	521	< 50	83.0	99.9
M326	57	6.0	280	< 50	66.9	112.9
M313	51	7.5	474	< 50	undetectable	undetectable
M309	56	2.9	359	< 50	3.8	5.5
M301	51	3.8	260	< 50	23.9	8.7
B013	24	2.5	580	< 50	34.8	65.9
W102	76	5.2	N/A	< 50	283.9	479.3
20 samples	**24-76**	**1.3-7.5**	**89-1582**	**< 50**	**+ 14/20, 4-284 copies/million PBMC**	**+ 12/20, 6-479 copies/million PBMC**

(a): CD4 count when collection (cells/mm3), (b): viral load when collection (copies/ml plasma), (c): cell-associated HIV-1 RNA copies per $1x10^6$PBMCs was determined by RT-ddPCR, (d): cell-associated HIV-1 RNA copies per$1x10^6$PBMCs was determined by real time RT-PCR. N/A: Medical records not available; undetectable: under 10 μl RNA (from 0.5 million PBMC) per reaction.

CONCLUSIONS

- RT-ddPCR accurately quantified ca-HIV-1 RNA transcripts; the dynamic range was 10 to 10^5 copies per 10 μl RNA input .
- Cell-associated HIV-1 RNA measured by RT-ddPCR and real time RT-PCR in samples from co-culture of serially diluted reactivated ACH2 cells together with MT2 cells lines were highly correlated (R^2=0.9668).
- Cell-associated HIV-1 RNA was detected by RT-ddPCR in 14/20 samples (range, 4-284 copies/million PBMC) and by real time RT-PCR in 12/20 samples (range, 6-479 copies/million PBMC), respectively.
- RT-ddPCR demonstrated similar accuracy and sensitivity compared to real-time RT-PCR in the measurement of cell-associated HIV-1 RNA in both cell-lines spiked with HIV-infected cells and in patient samples. RT-ddPCR is a promising tool for the quantification of HIV-1 cell-associated RNA.

ACKNOWLEDGMENTS

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