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Investigating Mechanisms of HIV Persistence using Droplet Digital PCR Approaches

Elizabeth M. Anderson[1], R. Gorelick[2], S. Hill[1], C. Rehm[3], M.F. Kearney[1], J.W. Mellors[4], J.M. Coffin[5], M. Piatak[2], and F. Maldarelli[1]

[1]HIV Drug Resistance Program, National Cancer Institute, Frederick, MD, [2]HIV Molecular Monitoring Core, Leidos Biomedical Research Inc., Frederick, Maryland,
[3]Laboratory of Immunoregulation, NIAID, Bethesda, Maryland, [4]Department of Medicine, University of Pittsburgh, Pittsburgh, PA, [5]Department of Molecular Biology and Microbiology, Tufts University, Boston MA

HIV Drug Resistance Program
NCI-Frederick

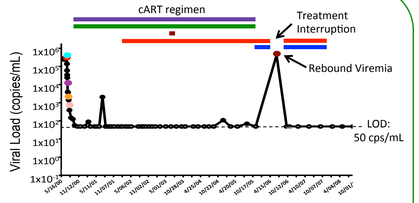
INTRODUCTION:

HIV persists at low levels in blood during antiretroviral therapy yet mechanisms of HIV persistence remain poorly characterized. In addition, the transcriptional activity of persistent proviruses is not understood and sensitive quantitative assays are needed to investigate this process. Droplet digital PCR (ddPCR) has been used to detect retrovirus sequences, especially HIV DNA, with single targets of 60-80 nucleotides, however optimal primer-probe combinations to detect the various HIV RNA transcripts have not been defined. We have developed new quantitative assays to explore mechanisms of HIV persistence by measuring its transcriptional activity.

BACKGROUND:

HIV Persistence:

- Despite success of cART, low level viremia persists for years after initiation
- Viremia rebounds to pretherapy levels if cART is interrupted.
- Source of persistent viremia is unknown



Characteristics of Cells infected with HIV:

- Distributed in a number of memory and naïve CD4 subsets
- Total numbers of infected cells decline after introduction of cART
- Can undergo clonal expansion via cell proliferation
- A subset produce HIV at low levels
- Are the source of rebound Viremia
- Can be reactivated in vitro

Potential Mechanisms of Persistence:

1. Cellular
   - Cells not activated, no transcription factors
   - Integrant with unfavorable chromatin structure
2. Viral
   - Proviral structure defective or deleted
   - Cells are activated, but virus is not

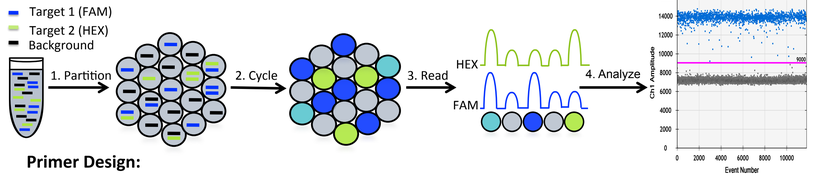
Hypothesis: Detecting HIV Nucleic Acid Species Will Shed Light on Mechanisms of Persistence

Mechanism	Possibilities	HIV Nucleic Acid Detection	Expected Quantification
No Transcription	Chromatin not properly phased or transcription factors not abundant	NO HIV RNA, HIV DNA present	Cannot measure RNA, only DNA
No Tat	Tat- from deletion or mutation of *tat* or TAR	Short transcripts	TAR:RU5:*gag* ratio of 1:0.5:<0.1 *based on Sharp 1991 data
No Rev/RRE	Rev- from deletion or mutation of *rev*/RRE	Transcripts are all spliced	TAR:RU5:*gag* ratio of 1:1:0
Tat and Rev Present	Active Transcription, copy number uncertain	Spliced and unspliced RNA	All RNA species, levels unknown

METHODS:

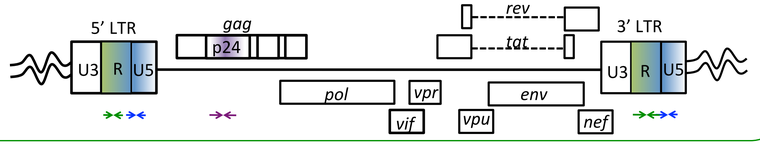
ddPCR Technology:

ddPCR quantifies DNA copy number by partitioning the PCR reactions into 20,000 nanoliter oil in water droplets (i.e., 20,000 separate reactions each containing a single molecule and a fluorescent-labeled probe), then by cycling the droplets to end point, and counting the number positive droplets with a FACS-like reader. This enables exact enumeration of amplifiable molecules in the starting sample.



Primer Design:

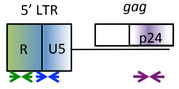
Designed Primers to quantify TAR (green), R-U5 (blue), and p24 of *gag* (purple). This will allow us to distinguish transcripts that are 58, 114, and 900 nt in length respectively. The system is flexible to allow multiplexing of pairs of primer probe sets with distinct fluorescence profiles. Additionally we will be able to quantify DNA equivalents by adapting these primers in conjunction with a CCR5 set.

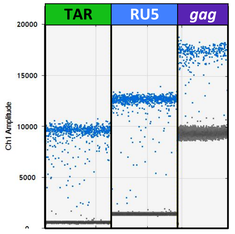


QUANTIFICATION OF HIV RNA:

RNA Transcripts:

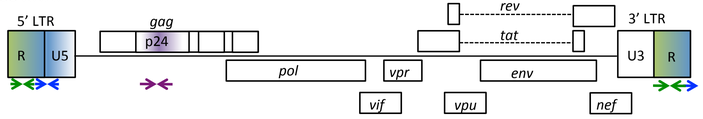
-1032 bp long RNA transcripts from +1 - 1032
-Expect 1:1:1 ratio or TAR: RU5: *gag*
-*Average Duplicates from 4 separate runs
-Accurately quantified down to 1 template/well





Platform/expt		TAR	RU5	gag
ddPCR 16,666	Ratio	0.87	1.16	0.63
	Measured*	14,454	19,404	10,495

Cell-Free RNA:



-RNA extracted from pNL4-3 virions
-Expect Ratio of 2:1:1 for TAR: RU5: *gag*
-*Averaged Duplicates from 3 runs

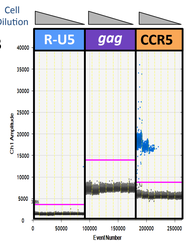
Platform/expt		TAR	RU5	gag
ddPCR 16,666	Ratio	1.93	1.03	1.53
	Measured*	33,160	17,185	25,450

QUANTIFICATION OF HIV DNA

Cell Culture:

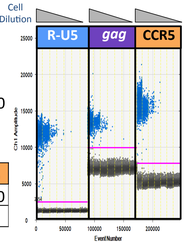
-Uninfected CEM cells
-CCR5 gene is located on chr 3
-Expect a ratio of 0:0:2 for RU5 : *gag* : CCR5
-Averaged duplicates 10,000 cell input from one run

	RU5	gag	CCR5
Avg.	0	0	18,300
Ratio	0	0	1.90



-ACH2 cells harbor a single integrated provirus in chr 7
-Expect a ratio of 2:1:2 for RU5 : *gag* : CCR5
-Averaged duplicates 10,000 cell input from one run

	RU5	gag	CCR5
Avg.	11,880	6,820	11,720
Ratio	2.00	1.00	1.80



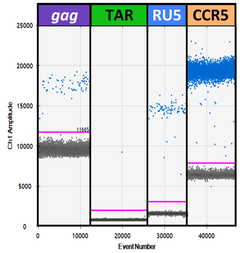
HIV Infected Patients:

-Chronically Infected
-Not on cART

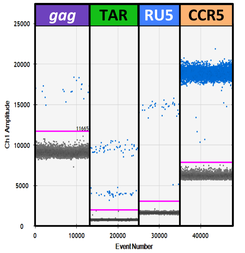
			Multiplexed				Single-plexed			
			gag		TAR		R-U5		CCR5	
PID	RNA (cps/mL)	CD4	Measured	Avg	Measured	Avg	Measured	Avg	Measured	Avg
Pt 4	584,204	7	378 284	331	8.68 27	17.84	902 948	925	79600 83000	81300
			1.00		**0.05**		**2.79**		**245.62**	
Pt 5	58,100	186	70.6 62.4	66.5	312 266	289	294 354	324	26800 25800	26300
			1.00		**4.35**		**4.87**		**395.49**	
Pt 9	202,989	299	65.6 51.6	58.6	172.2 206	189.1	168.8 116.8	142.8	39400 42000	40700
			1.00		**3.23**		**2.44**		**694.54**	

- **Bolded** is the ratio of TARGET (*gag*, TAR, RU5, or CCR5) to average gag measured

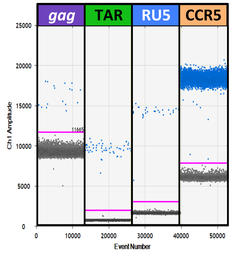
Patient #4



Patient #5



Patient #9



SUMMARY:

- ddPCR approaches distinguish HIV RNA transcription profiles in HIV infected cells
- Broad dynamic range (1-1x10^5 copies)
- Suitable for single cell analyses.
- Permit detailed and precise analysis of HIV transcription

FUTURE DIRECTIONS:

- Quantify RNA species in HIV-infected patients over time
- Adapt to detect integrant specific proviruses
  - measure the kinetics of proliferation of cells that give rise to clonal HIV populations over time.

REFERENCES:

- Mbonye, U., Karn, J. *Transcription control of HIV latency. Review.* 2014. Virology 454-455
- Maldarelli F et al. *Specific HIV integration sites are linked to clonal expansion and persistence of infected cells.* 2014. Science 345
- Ho, Y.C et al., *Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. 2013.* Cell 155(3)

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