A Novel ddPCR Protocol to Estimate Copy Numbers of Likely Intact HIV-1 Provirus

00311

Claire N Levy[1], Sean M Hughes[1], Pavitra Roychoudhury[2], Daniel B Reeves[2], Chelsea Spragg[2], Haiying Zhu[5], Meei-Li Huang[5], Yulun Wei[5], Marta E Bull[3], Noah AJ Cassidy[4], Jan McClure[5], Robert W Coombs[5], Dara A Lehman[4], Keith R Jerome[2,5*] & Florian Hladik[1,2,6*]

[1]Department of Obstetrics & Gynecology, University of Washington; [2]Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center; [3]Seattle Children's Research Institute; [4]Division of Human Biology, Fred Hutchinson Cancer Research Center; [5]Department of Laboratory Medicine, University of Washington; [6]Department of Medicine, University of Washington Seattle, WA

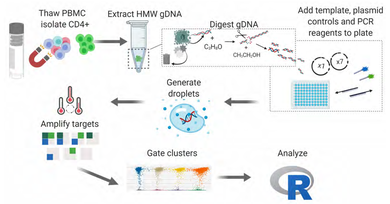
BACKGROUND

As the number of HIV cure studies increases, so does the need for a precise, specific, and sensitive method to quantify replication-competent proviral HIV DNA.

Accurately quantifying the replication-competent HIV reservoir is essential for evaluating the efficacy of HIV cure strategies. Ideally, this should be achieved by a rapid turn-around high-throughput assay suitable for a clinical setting

OVERVIEW OF METHODS

- We designed a multiplex ddPCR protocol to quantify potentially intact provirus in cells or tissues from ART-suppressed people living with HIV (PLWH).
- Our DNA isolation method for cells and tissue samples minimizes mechanical shearing.
- Optimized restriction enzyme digestion prepares gDNA for droplet formation while minimizing the amount of cutting within the viral genome.



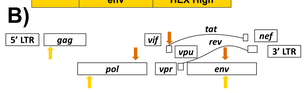
ASSAY DEVELOPMENT

- Our protocol targets 5 regions in the HIV genome across two ddPCR assays, each with 2 unique and 1 common target (**A, B**).
- The multiplex ddPCR allows us to assess potentially intact ("intact") proviral genomes by quantifying the number of droplets positive for 3 targets per assay (**C**, **D**).

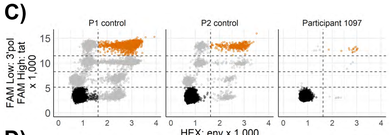
A)

Assay	Target	Dye
assay1	3'pol	FAM Low
	tat	FAM High
	env	HEX High
assay2	5'pol	FAM High
	gag	HEX Low
	env	HEX High

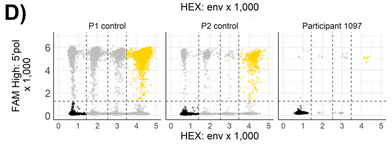
B)



C)

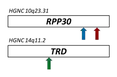


D)



- Our reference assay measures two targets in *RPP30* to quantify all cells and a target in the T cell receptor D gene (*TRD*), which is deleted during TCR rearrangement and therefore identifies all *non*–T cells. This allows normalization of HIV copy number per 1x10^6 T cells (**E**-**G**).

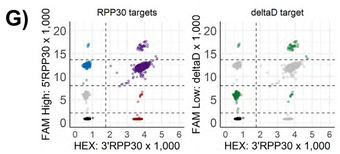
E)



F)

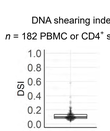
Assay	Target	Dye
Reference	deltaD	FAM Low
RPP30 and deltaD	5'RPP30	FAM High
	3'RPP30	HEX High

G)



- We calculated a DNA shearing index (DSI) and mathematically corrected for residual DNA shearing as measured by *RPP30* targets (**H**,**I**).

H)



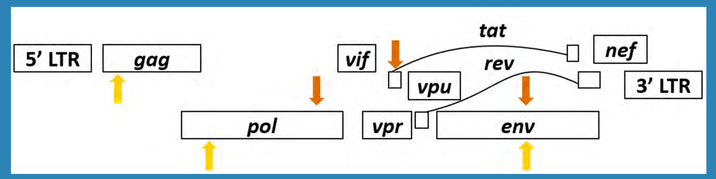
I)

$$\text{DSI} = \frac{1/2\,(S1 + S2)}{D + 1/2\,(S1 + S2)}$$

Where:
S1 = 5' RPP30 single positive droplets
S2 = 3' RPP30 single positive droplets
D = RPP30 double positive droplets

$$\text{corrected triple positive copies} = \frac{\text{observed triple positive copies}}{1 - \text{DSI}}$$

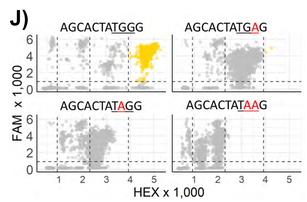
Our 5-probe ddPCR protocol quantifies intact HIV-1 provirus in cell and tissue samples



ASSAY VALIDATION

- Our method results in reduced shearing of DNA: average DSI = 0.12
- Limit of blank: 0 triple positive copies/10^6 cells
- Limit of detection: 96.1 triple positive copies/10^6 cells assayed
- Reproducibility (repeated runs of same sample):
  - assay1: %CV 42.4; assay2, %CV 42.5
- G > A hyper-mutation in probe binding site results in "defective" call

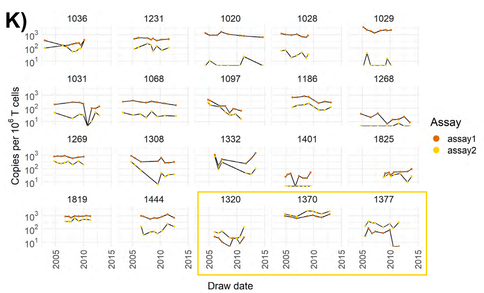
J)



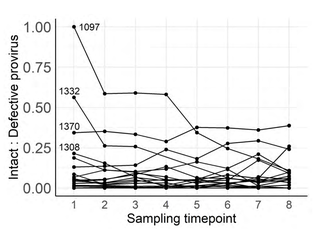
APPLICATION TO CLINICAL SAMPLES

- We applied this protocol to 157 CD4$^+$ T cell samples from a longitudinal cohort of 20 PLWH (**K**).
  - assay1: mean, 538.8 copies/10^6 T cells 95% CI 449.2-628.1.
  - assay2: mean, 186.4 copies/10^6 T cells 95% CI 126.7-246.2.
  - assay1 reported higher results for 135/157 (86%) of samples.
  - assay1 was consistently higher in 17/20 participants and assay2 was consistently higher in 3/20 participants (**K**, yellow box).
  - We calculated an average half life of 55 months (based on lower assay result).
- The ratio of intact:defective provirus shows variable kinetics between participants over time (**L**) . These results can be compared to viral loads, CD4$^+$ T cell counts and other clinical measures.

K)



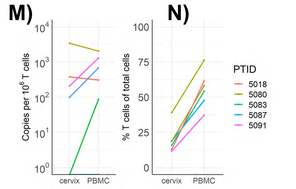
L)



APPLICATION TO CLINICAL SAMPLES (CONTINUED)

- We applied our protocol (assay2 and reference only) to 6 paired PBMC and cervical biopsy samples from PLWH with a range of plasma viral loads (**M**).
  - PBMC: mean: 738.5/10^6 T cells
  - Cervix: mean: 683.3/10^6 T cells
  - We used the deltaD assay to quantify percent T cells from blood and tissues. Means were significantly different between tissue and PBMC, α= 0.05, p = 1x10^{-4} (Welch's test for unequal variances) (**N**).

M) **N)**



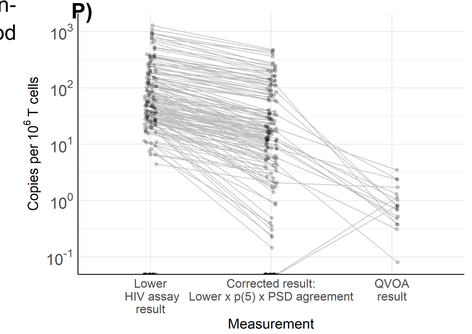
ESTIMATION OF TRUE INTACTNESS AND COMPARISON TO QVOA

- We calculated the probability that a sample was called intact by *both* assay1 and assy2 (i.e. positive for 5 targets), and applied this probability, "p(5)", to DSI-corrected assay results to adjust for over-estimation of intact copies.
- An *in silico* assessment of clade B sequences available from the Proviral Sequence Database (PSD) showed 90.2% agreement in "intact vs defective" calls between our method and the PSD algorithm (**O**).
- We applied p(5) and PSD correction to the lower result of the two assays and compared it to the uncorrected result in 175 blood CD4+ T cell samples (**P**).
- In 15/175 samples, we compared those values to the infectious units per million from a QVOA (**P**).

O)

	Count of sequences defective by ddPCR	Count of sequences intact by ddPCR
Count of sequences defective by PSD	**910**	94
Count of sequences intact by PSD	11	**56**

P)



CONCLUSIONS

- Our protocol to quantify potentially intact HIV provirus is specific, sensitive, reproducible and applicable to cell and tissue samples.
- Multiple targets can be detected simultaneously, which increases the likelihood that detected sequences originated from same cell and virus.
- It is theoretically possible that HIV target sequences from different cells could be partitioned into the same droplets, but, due to the low number of proviral copies, this is very unlikely.
- Although our assay may still overestimate reservoir size due to defective regions outside of the target sequences, but the 5-target design improves our ability to quantify intact proviruses compared to ddPCR assays using fewer regions.

Author Contact Information
Claire Levy: clairel@uw.edu
Florian Hladik*: fhladik@fredhutch.org
Keith Jerome*: kjerome@fredhutch.org
*These authors contributed equally

Acknowledgements

We thank study participants for their generous support of this work.

This work was supported by a grant from the James B. Pendleton Charitable Trust.

Research reported in this publication was supported by the University of Washington / Fred Hutch Center for AIDS Research, an NIH-funded program under award number AI027757 which is supported by the following NIH Institutes and Centers: NIAID, NCI, NIMH, NIDA, NICHD, NHLBI, NIA, NIGMS, NIDDK. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.