NIH NATIONAL CANCER INSTITUTE

0561

In-depth Evaluation of the Intact Proviral DNA Assay for Quantification of the HIV-1 Reservoir

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BACKGROUND

- Accurate quantification of the HIV-1 reservoir is critical in understanding HIV-1 persistence and evaluating cure strategies [1].
- The Intact Proviral DNA Assay (IPDA), a droplet digital PCR assay that simultaneously targets the HIV packaging signal (psi) and *env* regions, is widely used to quantify the intact or rebound-competent reservoir, although the effect of intra-patient genetic diversity on IPDA results has not been extensively investigated.
- Several adaptations, such as increasing the number of HIV targets or combining with long-range amplification, have been suggested for the IPDA [3-6].
- We assessed the precision of IPDA in distinguishing intact from defective proviruses using genetically characterized single genome sequences (SGS).

METHODS

- Genomic DNA was extracted using an in-house guanidine-based method to preserve DNA quality from the peripheral blood of 16 persons with HIV (PWH) at pretherapy (n=10) and multiple time points on ART (n=29).
- For 13/16 PWH with successful IPDA, we obtained a total of 5782 single-genome proviruses by amplification using primers targeting the 5' and 3' LTR of HIV-1 as previously described [7]. PWH with IPDA failure were not included for further comparison.
- We sequenced and analyzed 1091 near-full length (NFL) SGS >7kb in Geneious Prime 2025.0.2 and genetically classified them as intact or defective (479 intact and 612 defective sequences) (https://psd.cancer.gov/tools/pvs_annot.php)
- Corresponding PCR products of >7kb single genomes were diluted and submitted to real-time amplification (qPCR) using IPDA primers/probes [2]. A subset of proviruses < 7kb was not sequenced but also submitted for qPCR using IPDA primers/probes [2].

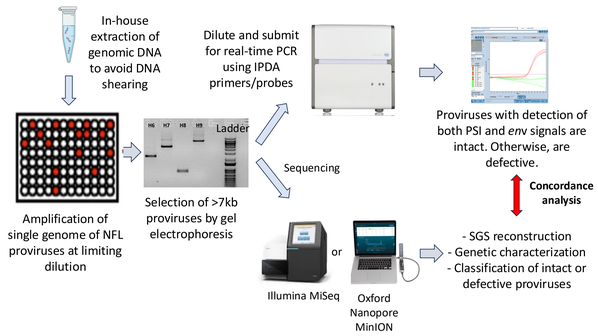


Figure 1: Schematic experimental procedure

RESULTS

Table 1: Participant characteristics

Characteristics	Median (interquartile range)
Age (years)	61 (58-67)
Sex	Male (87.5%)
CD4 count (cells/ul) **- Pretherapy (n=10)** **- On ART (multiple timepoints, n=29)**	 259 (166.5-466.3) 581 (367-714)
Time on suppressive ART (to the latest sample (years))	14 (8.8-18.3)

IPDA is generally accurate in distinguishing intact from defective proviruses, although error rates may vary among samples from PWH with highly genetically diverse HIV. Careful interpretation of the IPDA results is essential.

1) In silico analysis of Near-full-length proviruses revealed significant mismatches of intact proviruses with IDPA primers and probes

- Sequences of IPDA primers/probes were mapped to **702 intact** and **4931 defective** sequences retrieved from literature (https://psd.cancer.gov/tools/pvs_annot.php).
- Only 30% of intact proviruses matched identically with the PSI and RRE primers and probes set of IPDA, suggesting **the possible underestimation of intact proviral level**.
- A small proportion of >7kb defective proviruses harbored no mismatch with IPDA primers and probes, suggesting **a very low level of misclassification of defective as intact proviruses**.
- All defective proviruses < 7kb harbored significant deletion in the IPDA primers/probes binding areas.

Table 2: Frequency of proviruses harbored 0-2 mismatches with IPDA primers/probes

Number of mismatches per IPDA primer/probe	Intact (n, %)	Defective (n, %)	Defective ≥ 7kb (n, %)	Defective <7kb (n, %)
0 mismatch	208 29.63%	8 0.16%	8 0.31%	0 0%
1 mismatch	369 52.56%	254 5.15%	254 9.98%	0 0%
2 mismatches	499 71.08%	464 9.4%	464 18.3%	0 0%

2) Experimental evaluation of IPDA using amplified products of single-genome proviruses

2a) Varying rates of misclassification by IPDA from pretherapy to ART and across PWH (table 3)

- Per total amplified single-genome proviruses, we observed varying misclassification rates of intact and defective proviruses by IPDA across timepoint and participants.

Table 3: Misclassification rates by IPDA

Timepoint	Proportion of Intact misclassified as defective per total proviruses	Proportion of >7kb defective misclassified as intact per total proviruses	Proportion of <7kb defective misclassified as intact per total proviruses
Pretherapy	0% (0-23.3)	4.2% (0-14.8%)	Not done
On ART	0% (0-7.7%)	1.8% (0-9.4%)	8.8% (2.0-27.3)
P-value	ns	**0.001**	Not applicable

2b) Intra-individual diversity of intact proviruses in PSI region may lead to biased quantification of the intact level

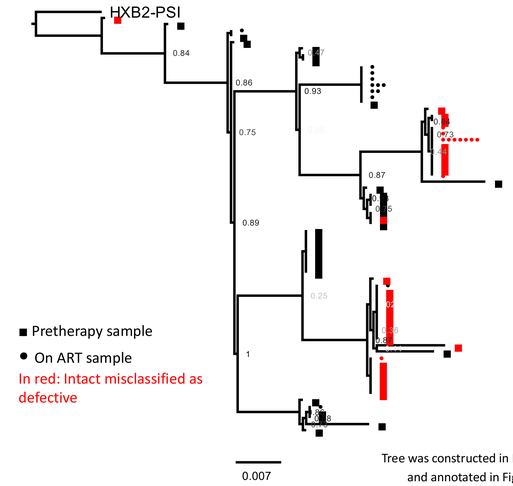


Figure 2: Phylogenetic tree of PSI region (position 692-798, similar to IPDA targeted region) derived from intact sequences obtained at pretherapy and on ART in one participant.

The tree shows nucleotide diversity in the intact proviral populations in the PSI region. Intact sequences misclassified as defective (in red) are clustered together. Analysis of misclassified intact sequences revealed the mismatches/deletion at **the last two nucleotide positions** (757-758 compared to HXB2) corresponding to the IPDA PSI probe binding site are determinants for the failure of IPDA.

2c) Defective proviruses misclassified as intact harbored mutations both in and outside IPDA-targeted regions

- Sequencing analysis of 158 defective proviruses >7kb misclassified as intact revealed mutations **both inside** (15.8% in psi) and **outside IPDA** targeted regions (19.6% in *gag*, 24.7% in *pol*, 29.7 in *env*). Interestingly, the **RRE status** (IPDA targeted region on *env*) **is good** for all misclassified defective proviruses.
- <7kb defective proviruses (not sequenced), even with large internal deletion, can still harbor intact PSI and *env* regions detected by IPDA, hence, being misclassified as intact proviruses. The misclassification rate varied across participants (table 3).

CONCLUSIONS

- IPDA is generally accurate in distinguishing intact from defective, but the error rate varies from pretherapy to ART and across PWH.
- Of note, intra-individual intact proviral populations are diverse and can lead to erroneous quantification of the intact level within an individual across different time points.
- Careful interpretation of the IPDA results is essential, especially in evaluating interventions with minor changes in the intact proviral level.
- If the intact proviral level by IPDA is the main end-point of evaluation strategies, low-coverage single genome sequencing of IPDA-targeted regions (PSI and *env)* is helpful to evaluate the rate of bias due to proviral diversity.

ADDITIONAL KEY INFORMATION

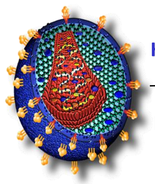
Reference

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Acknowledgments: ALL THE STUDY PARTICIPANTS



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