



Intact proviral DNA assay analysis of large cohorts of people with HIV provides a benchmark for the frequency and composition of persistent proviral DNA

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A scalable approach for quantifying intact HIV-1 proviruses is critical for basic research and clinical trials directed at HIV-1 cure. The intact proviral DNA assay (IPDA) is a novel approach to characterizing the HIV-1 reservoir, focusing on the genetic integrity of individual proviruses independent of transcriptional status. It uses multiplex digital droplet PCR to distinguish and separately quantify intact proviruses, defined by a lack of overt fatal defects such as large deletions and APOBEC3G-mediated hypermutation, from the majority of proviruses that have such defects. This distinction is important because only intact proviruses cause viral rebound on ART interruption. To evaluate IPDA performance and provide benchmark data to support its implementation, we analyzed peripheral blood samples from 400 HIV-1⁺ adults on ART from several diverse cohorts, representing a robust sample of treated HIV-1 infection in the United States. We provide direct quantitative evidence that defective proviruses greatly outnumber intact proviruses (by >12.5 fold). However, intact proviruses are present at substantially higher frequencies (median, 54/10⁶ CD4⁺ T cells) than proviruses detected by the quantitative viral outgrowth assay, which requires induction and in vitro growth (~1/10⁶ CD4⁺ T cells). IPDA amplicon signal issues resulting from sequence polymorphisms were observed in only 6.3% of individuals and were readily apparent and easily distinguished from low proviral frequency, an advantage of the IPDA over standard PCR assays which generate false-negative results in such situations. The large IPDA dataset provided here gives the clearest quantitative picture to date of HIV-1 proviral persistence on ART.

IPDA | HIV | reservoir | latency | cure

In individuals with HIV-1, a latent form of the virus persists in resting CD4⁺ T cells despite effective antiretroviral therapy (ART) that suppresses the level of free virus in the plasma to below the limit of detection of clinical assays (1–3). This latent reservoir is a major barrier to cure and the subject of an intense international research effort. Accurate measurement of the reservoir is essential for evaluating cure strategies (4). The reservoir was originally defined using a quantitative viral outgrowth assay (QVOA) for latently infected cells that release replication-competent virus after reversal of latency by a single round of in vitro T cell activation (1). This assay provides a definitive minimal estimate for the frequency of latently infected cells; however, it is costly, requires large blood volumes, and misses many replication-competent proviruses that are not induced following a single round of T cell activation (5, 6). In addition, an elegant recent study has shown that extinction events in culture may lead to a further underestimation of the total number of replication-competent proviruses (7).

Owing to the complexity of the QVOA, most studies use simpler PCR assays that measure proviral DNA (4). However, these assays substantially overestimate reservoir size because they do not

distinguish intact HIV-1 proviruses from the vast excess of proviruses with overt fatal defects, including large deletions and/or APOBEC3G-mediated hypermutation (8, 9). Other assays measure viral RNA or protein expression before or after a single round of T cell activation (10–13); however, these assays not only miss proviruses not induced by a single round of T cell activation (as with the QVOA), but also may detect defective proviruses still capable of generating both viral RNA and proteins, leading to overestimation of reservoir size (14, 15).

A novel approach to reservoir analysis is the quantification of genetically intact proviruses irrespective of their transcriptional status at any given time. This can be done by near-full-length proviral sequencing (5, 8, 16–18), but such single genome sequencing approaches are not scalable. In addition, they do not provide absolute quantitation because they depend on an initial near-full-length (9 kb) PCR, which is inefficient. The recently described intact proviral DNA assay (IPDA) provides a scalable

Significance

Despite clinically effective antiretroviral therapy (ART), replication-competent HIV-1 persists as latent proviral DNA capable of rekindling viral replication when ART is stopped. To cure the infection, therapies are being developed to eliminate latent HIV-1. Accurate assays for intact or rebound-competent HIV-1 are important to this effort. We previously developed the intact proviral DNA assay (IPDA) as a more accurate and scalable assay for intact HIV-1 proviruses. Here we present IPDA analysis in a diverse cohort of 400 ART-treated individuals. We confirm that intact proviruses, although vastly outnumbered by defective proviruses, are present at significantly higher frequencies than previously detected by viral outgrowth assays. We also show that IPDA amplicon signal issues, observed in 6.3% of samples, result from sequence polymorphisms.

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way to determine the frequency of cells carrying intact proviruses (9). The IPDA leverages droplet digital PCR to simultaneously interrogate individual proviruses with two strategically chosen and highly informative short amplicons placed in conserved regions of the proviral genome. Interrogation at these regions enables the assay to distinguish and separately quantify apparently intact proviruses from the overwhelming majority of defective proviruses, which harbor large deletions and/or hypermutation.

The IPDA has already led to novel insights. For example, the assay has been used to provide more accurate estimates of the decay rate of the latent reservoir (19), to demonstrate differential decay rates for cells carrying intact and defective proviruses (9, 19, 20), to document low reservoir size in elite suppressors (21), and to explore the frequency and inducibility of intact HIV-1 proviruses in different subsets of CD4⁺ T cells (22).

The analysis of large and diverse cohorts is an important step in defining the performance of a novel assay. However, such systematic analysis has not been widely undertaken for assays for persistent HIV-1, including the IPDA. Owing to its scalability and sensitivity, the IPDA can be applied to large donor cohorts. In this study, we present the IPDA analysis of samples from a large and diverse group of individuals with HIV-1 who are on ART. This dataset will serve as a quantitative benchmark for the frequency and composition of proviral DNA in treated individuals. In addition, this analysis of a large group of people with HIV-1 defines the frequency with which HIV-1 sequence polymorphisms interfere with amplification in the IPDA, which has rarely been defined for other molecular assays for HIV-1 persistence. Furthermore, in this study we describe how the design of the IPDA allows for the detection of such amplification problems, which is not possible with other typical PCR-based approaches for quantifying proviral DNA. We also describe how these problems can be mitigated to determine the frequency of cells with intact proviruses. We suggest that this information will further support the implementation of a novel and scalable approach for measuring intact HIV-1 proviruses in cure studies.

Results

Independent Cohorts Included in This Study. We evaluated 400 individuals with HIV-1 who are on ART, making this one of the largest cross-sectional analyses of intact and defective proviruses to be undertaken. This effort required significant collaboration from multiple independent groups. A summary of the source of samples is provided in Table 1. All participants included in the study were HIV-1 positive adults on ART living in the United States who had suppression of detectable viremia for >6 mo. The aggregated study population was diverse with respect to age, sex, race, HIV risk factor, and other clinical parameters and thus represents a reasonable sample of treated HIV-1 infection in the United States. Demographic data for each contributing cohort are provided in Table 1.

Overview of the IPDA. An overview of the IPDA is provided in Fig. 1. DNA isolated from donor samples is distributed into nanoliter-size droplets such that individual proviruses are contained within single droplets, enabling multiplex analysis of each provirus. In each droplet containing a provirus, duplex PCR reactions occurring in each droplet interrogate two carefully chosen regions of the HIV-1 genome to distinguish intact from defective proviruses (Fig. 1*A* and *B*). These regions are the packaging signal (Ψ) and the Rev-response element of the *env* gene (9). Previous studies have shown that the majority of defective proviruses harbor large internal deletions (average size, 49.6% of genome) (9), and thus intact proviruses can be distinguished from most deleted proviruses based on successful amplification at just two positions in the genome. Amplification occurs in nanoliter-sized droplets containing all of the components necessary for the duplex PCR reactions (Fig. 1*C*). The intact proviruses are positive for both the

Ψ and *env* amplicons. In addition to large internal deletions, defective proviruses are often hypermutated, predominately by APOBEC3G (5, 8, 9, 25, 26). To distinguish hypermutated proviruses, the *env* amplicon targets a region containing adjacent, frequently mutated APOBEC3G consensus sites and uses labeled wild-type and unlabeled hypermutated probes. As a result, the majority of hypermutated proviruses fail to give a signal for the *env* amplicon (9).

In the IPDA, digital droplet PCR (ddPCR) results are displayed on a 2D dot plot with fluorescence from the Ψ and *env* probes on the *y*- and *x*-axes, respectively. Measurement of intact proviruses with the IPDA depends on the presence of droplets in all four quadrants of the plot (Fig. 1*D*). Proviruses with 3' deletions encompassing the *env* amplicon and hypermutated proviruses give a fluorescent signal only for the Ψ amplicon and are found in the upper left quadrant (Q1). Proviruses with 5' deletions encompassing the Ψ region give a signal only for the *env* amplicon and are found in the lower right quadrant (Q4). Droplets with no proviruses occupy the lower left quadrant (Q3), along with a small number of proviruses defective at both amplicons. Intact proviruses are counted directly as double-positive droplets in the upper right quadrant (Q2). In infected individuals who started ART during chronic infection, defective proviruses greatly outnumber intact proviruses. This is reflected in the typical droplet distribution observed in an IPDA 2D dot plot: Q3 (empty) >>> Q1 ~ Q4 > Q2. Data demonstrating the sensitivity, specificity, and reproducibility of the assay have been reported previously (9). As described previously, a separate ddPCR for a cellular gene quantitates input cell number and allows correction for DNA shearing (9).

IPDA Analysis of Peripheral Blood Samples from Individuals with HIV-1 on ART. In this study, IPDA was performed on DNA isolated from peripheral blood CD4⁺ T cells from 400 individuals with HIV-1 who were on ART. The number of CD4⁺ T cells analyzed per sample varied depending on initial cryopreserved cell availability and the number of total CD4⁺ T cells recovered (Fig. 2*A*). For most samples, $\sim 6 \times 10^5$ cells were analyzed. The output value of intact proviruses/10⁶ cells showed little or no dependence on input cell number (Spearman's $\rho = -0.1551$) or the degree of DNA shearing (Spearman's $\rho = 0.18$).

A summary of the proviral frequencies measured by the IPDA is shown in Fig. 2*B*. The median proviral frequencies per million cells as measured by the IPDA in this study were as follows: intact, 54; 3' defective, 322; 5' defective, 269; and total (calculated on a per donor basis), 755. The results are consistent with IPDA values reported initially from a smaller cohort (9). There were no significant differences in the frequency of intact proviruses between the groups in Table 1 using one-way ANOVA with Tukey's multiple-comparisons test. Analysis of this large cohort revealed that the frequencies of intact and defective proviruses can vary across a multi- \log_{10} range. Based on our previous analysis with near-full-length genome sequences (9), we predicted that only 4% of proviruses are likely to have defects at both IPDA amplicons and thus would not be directly quantified by the IPDA. In other words, we predict that 96% of proviruses would be captured and scored as intact or defective by the IPDA. Further analysis of the proviral landscape is needed to verify this estimate.

The relative composition of the population of persistent proviruses as measured by the IPDA is shown in Fig. 2*C*. Intact proviruses composed 8% of the total proviruses on average. Thus, our results provide direct evidence from a large diverse population of infected individuals that the frequency of cells with intact proviruses is substantially smaller than the frequency of cells with defective proviruses.

Previous analyses of the proviral landscape in ART-treated individuals with HIV-1 have relied on near-full-length genome sequencing, an approach that is not quantitative, due in part to the inefficiency of the long-distance PCR reactions involved (5, 8, 16–18). In contrast, the IPDA provides direct quantitation

Table 1. Summary of the source of samples in this study

Sample source	Institution	N*	Age, y, median	Male sex, %	Race, % [†]	Risk [‡]	ART duration, y	CD4 count, cells/ μ L, median		Reference
								Nadir	Proximal	
A5321/A53415	AIDS Clinical Trials Group	74	46	73.0	W 50 B 20 H 30	Mix	7.1	319	748	R. Gandhi et al. ⁵
SCOPE	University of California, San Francisco	81	49	95.1	W 74 B 12 H 8 O 6	MSM (>84%)	10.8	183	584	(19)
BEAT-HIV	Wistar Institute	63	44	89.0	W 14 B 79 O 7	Mix	>1	NA [¶]	786	(23)
ALIVE	Johns Hopkins	96	53	70.8	W, H 4 B 96	IVDU	7.8 [#]	180	464	G. D. Kirk et al.
Bartlett Clinic	Johns Hopkins	20	59	63.6	W 9 B 91	Mix	5.0	NA [¶]	670	(21, 24)
ACDX sample collection	AccelvirDx	66	56	73.5	W 18 B 72	Mix	9.3	NA [¶]	746	This study
Total		400								

*N, number of unique donors.

[†]W, White non-Hispanic; B, Black non-Hispanic; H, Hispanic (regardless of race); O, other.

[‡]Predominant risk factor for HIV-1 acquisition for the cohort. Mix, cohort includes participants with different risk factors for acquisition; MSM, men who have sex with men; IVDU, intravenous drug use.

⁵R. Gandhi et al., Conference on Retroviruses and Opportunistic Infections, March 8–11, 2020, Boston, MA.

[¶]NA, not available; data on CD4 nadir not available from patient records.

[#]Time since first introduction of ART. Due to nonstructured treatment interruption, the time from last detectable HIV-1 RNA was 3.4 y.

^{||}G. D. Kirk et al., Conference on Retroviruses and Opportunistic Infections, March 8–11, 2020, Boston, MA.

and the scalability to interrogate large cohorts. Our results also establish in a large and diverse population of HIV-1–positive individuals that intact proviruses are present at a substantially higher frequency than that observed in the QVOA (mean frequency, $\sim 1/10^6$ CD4⁺ T cells) (27–29). This difference reflects the fact that most intact proviruses are not induced following a single round of in vitro T cell activation (5, 6, 22). In addition, some cells carrying intact proviruses do not give rise to a sufficient amount of virus to establish a spreading in vitro infection (7). Finally, we have reported that some proviruses characterized as intact by the IPDA contain minor defects affecting viral fitness (9).

Considering these factors and taking advantage of the direct quantitation provided by the IPDA, we provide an updated picture of the proviral landscape in 400 individuals with HIV-1 receiving ART (Fig. 2D). Proviruses scored as intact are present at a frequency of 50 to 60/10⁶ CD4⁺ T cells, 12.5-fold less than the total frequency of infected cells and more than 50-fold greater than the frequency of cells detected by QVOA in previous studies (1, 4, 20–22). This is consistent with multiple previously published near-full-length proviral sequencing studies demonstrating that the QVOA underestimates the size of the latent reservoir (5, 8, 16–18). We observed a cross-sectional association between the frequency per million CD4⁺ T cells of intact proviruses and total proviruses (Spearman's $\rho = 0.76$; $P < 0.0001$). The ratio of intact to total proviruses for the aggregate dataset showed considerable interparticipant variability (Fig. 2E), consistent with published analysis for the SCOPE subgroup (19).

IPDA Amplicon Signal Failure due to Polymorphism Is Infrequent and Readily Distinguished. For all PCR-based HIV assays, sequence variation in the primer or probe binding sites can prevent the

generation of a signal in samples from some individuals. The frequency at which this occurs has not been systematically investigated for commonly used molecular assays for persistent HIV-1. The IPDA uses primers and probes designed to bind to conserved regions of the HIV-1 genome, based on clade B alignments (9). The ddPCR format and the primer/probe design are tolerant of minor sequence mismatches. Nevertheless, within large populations with HIV-1, some amplification problems due to sequence polymorphism are expected.

Importantly, the design of the IPDA allows such problems to be immediately identified. Polymorphisms preventing signal generation from the Ψ amplicon will result in plots with no droplets in Q1 and no droplets in Q2 (Fig. 1D). Similarly, polymorphisms preventing signal generation from the *env* amplicon will result in plots with no droplets in Q2 and no droplets in Q4. In such situations, intact proviruses cannot be quantitated unless alternative primers or probes are used (see below). Except in rare cases of failure of both amplicons, the two-amplicon format of the assay automatically distinguishes signal problems from the absence of proviral templates, thus preventing the reporting of false-negative results. This is another important advantage of the IPDA over single-amplicon quantitative PCR assays for proviral DNA, which cannot identify poor amplification or signal failure due to polymorphisms.

In this study, we evaluated signal failure by identifying samples for which no fluorescence-positive droplets were discernible for one of the amplicons, while the other amplicon exhibited normal amplification and droplet fluorescence. Of note, signal failure has not been evaluated for any other PCR-based reservoir assay. In this cohort of 400 individuals with HIV-1, samples from 3.5% of individuals exhibited signal failure in the Ψ amplicon, and

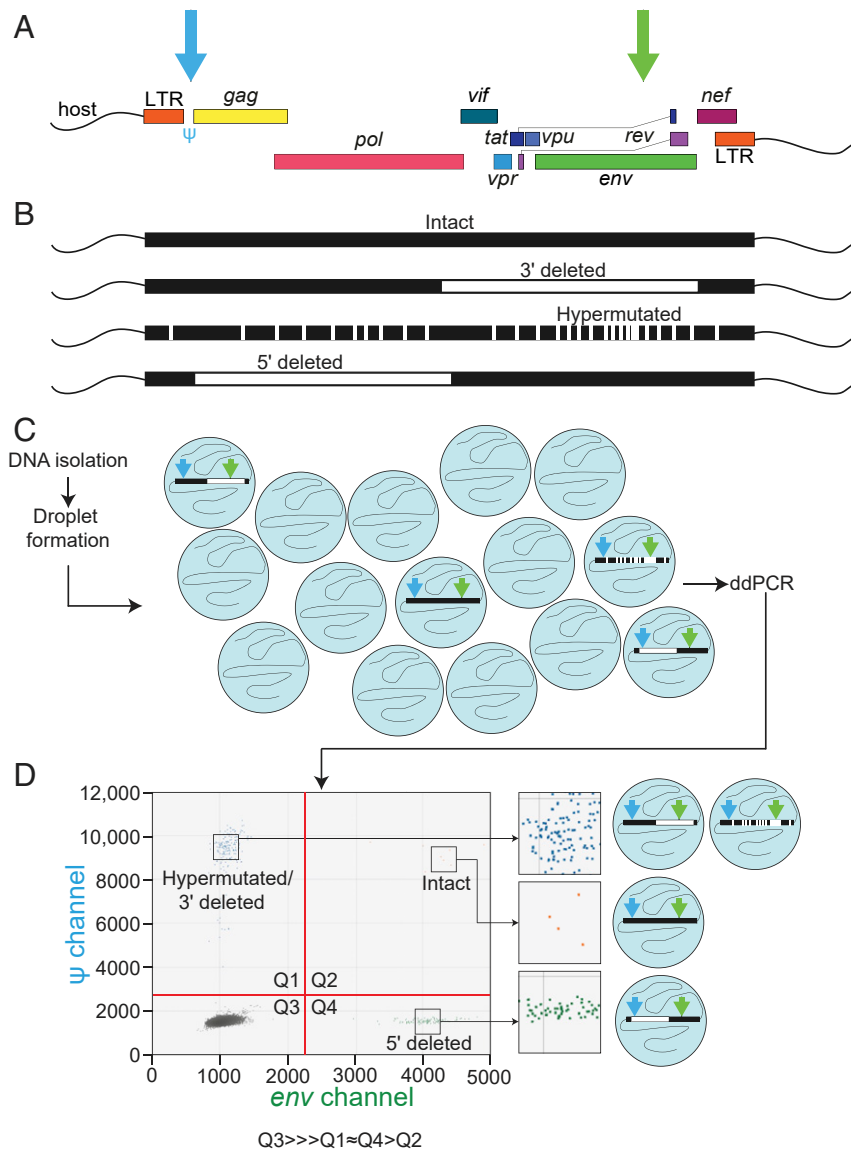


Fig. 1. Overview of the IPDA. (A) Map of the HIV-1 genome indicating positions of the Ψ (blue arrow) and *env* (green arrow) amplicons. (B) Illustration of the common types of fatal defects detected by the IPDA. Precision maps of sequenced proviruses are available elsewhere (5, 8, 9). (C) Overview of the IPDA. In addition to the HIV-1 proviral discrimination reaction illustrated here, the IPDA includes a parallel ddPCR reaction targeting a host gene to allow determination of input cell number and correction for shearing between Ψ and *env* amplicons (9, 19). (D) Representative 2D ddPCR plot from the HIV-1 proviral discrimination component reaction of the IPDA. The typical droplet counts follow the pattern $Q3 \gg Q1 \sim Q4 > Q2$.

samples from 2.8% of individuals exhibited signal failure in the *env* amplicon (Fig. 3A), while 93.7% of the samples from 400 donors exhibited no signal issues. Examples of signal failures are shown in Fig. 3B and C. As each IPDA run included both positive controls (J-Lat 6.3 cells) and negative controls ($CD4^+$ T cells from HIV-1-negative donors), we ensured that all assay runs were successful in specifically amplifying both the Ψ and *env* regions of control HIV-1 proviral DNA.

Using independent longitudinal samples collected several years apart, we confirmed that signal failures were consistently observed within the same donor. For example, in two donors (04036 and 04022) whose samples exhibited signal failure in the Ψ amplicon, signal failure was consistent across two timepoints separated by multiple years of suppressive ART. The IPDA plot for donor 04036 is shown in Fig. 3B. In addition, we performed targeted amplicon sequencing on samples from selected donors with signal failures. In these samples, we observed polymorphisms

in the IPDA oligo-binding sites, which would account for signal failure. In each of these samples, we identified the source of amplification failure as a polymorphism at the critical 3' end of the forward Ψ primer that is predicted to prevent PCR extension (Fig. 3D). Similarly, for donors 04052 and 04122, whose samples showed signal failure for the *env* amplicon, we identified polymorphisms which could account for that signal failure (Fig. 3E-G).

In cases where sequence polymorphisms cause signal failure for the Ψ or *env* amplicons, minor adjustments in the primer or probe sequences can restore the signal, producing the expected droplet pattern ($Q3 \gg Q1 \sim Q4 > Q2$) and allowing for quantitation of intact proviruses. An example is shown in Fig. 4.

Discussion

In this large multicohort study, we used a novel reservoir assay, the IPDA, to provide a quantitative picture of the landscape of persistent proviruses that preclude the cure of HIV-1 infection

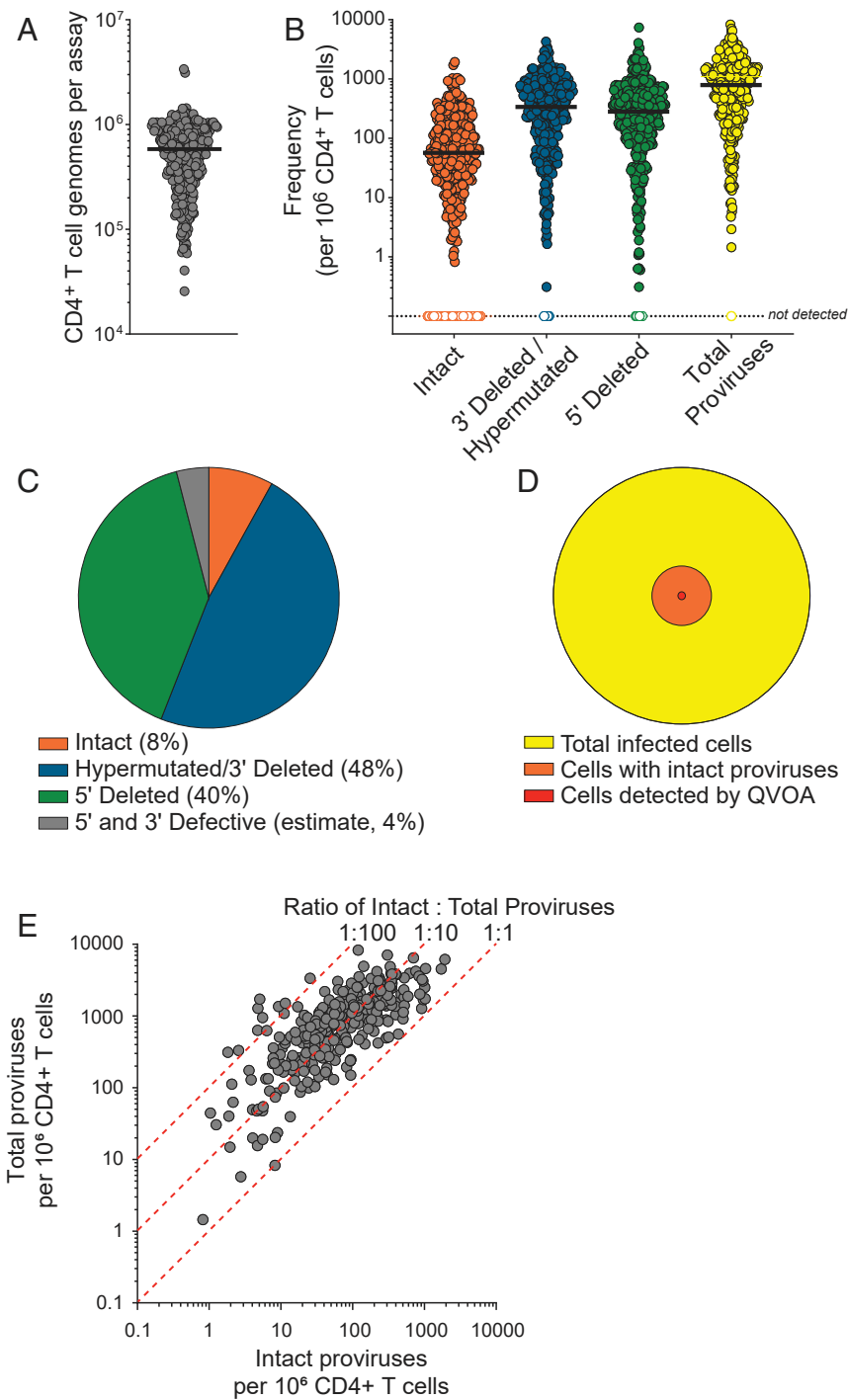


Fig. 2. IPDA analysis of peripheral blood CD4⁺ T cells from 400 HIV-1-infected individuals on ART provides absolute frequencies and relative composition of the population of persistent proviruses. (A) Number of CD4⁺ T cell genomes analyzed per assay, as measured by the copy reference/DNA shearing component reaction of the IPDA. The horizontal line indicates median value. $n = 400$ (B) Absolute frequencies of intact and defective proviruses per 10⁶ total CD4⁺ T cells as measured by the IPDA. Total proviruses were determined as the sum of intact, 5' defective, and 3' defective proviruses from each donor. Horizontal line indicated median values. $n = 400$ (C) Average relative composition of persistent proviruses as measured by the IPDA. (D) Landscape of persistent proviruses in ART-treated individuals with HIV-1. The QVOA detects an average of 1/10⁵ resting CD4⁺ T cells, based on previous studies (1, 4, 20–22). The value for the frequency of cells with intact proviruses (38/10⁶ resting CD4⁺ T cells) is based on the mean frequency measured in B, corrected for the fraction of cells with small defects not detected by the IPDA (~30% of Q2 droplets) (9). The value for total infected cells is based on data from B, corrected for the fraction of proviruses (4%) not detected by either of the IPDA amplicons. (E) Frequency of intact and total proviruses per million CD4⁺ T cells, as measured by the IPDA. The dashed lines reference relative ratios of intact:total proviruses. Correlation was performed on log-transformed data (Spearman's $\rho = 0.76$; $P < 0.0001$).

with ART. Previous studies using near-full-length sequencing of individual proviruses showed that the landscape of persistent proviruses is dominated by those with very large deletions and/or

APOBEC3G-mediated hypermutation (5, 8, 9, 16–18). Most of these defective proviruses are defective in most viral genes (9) and thus cannot contribute to viral rebound, and these should

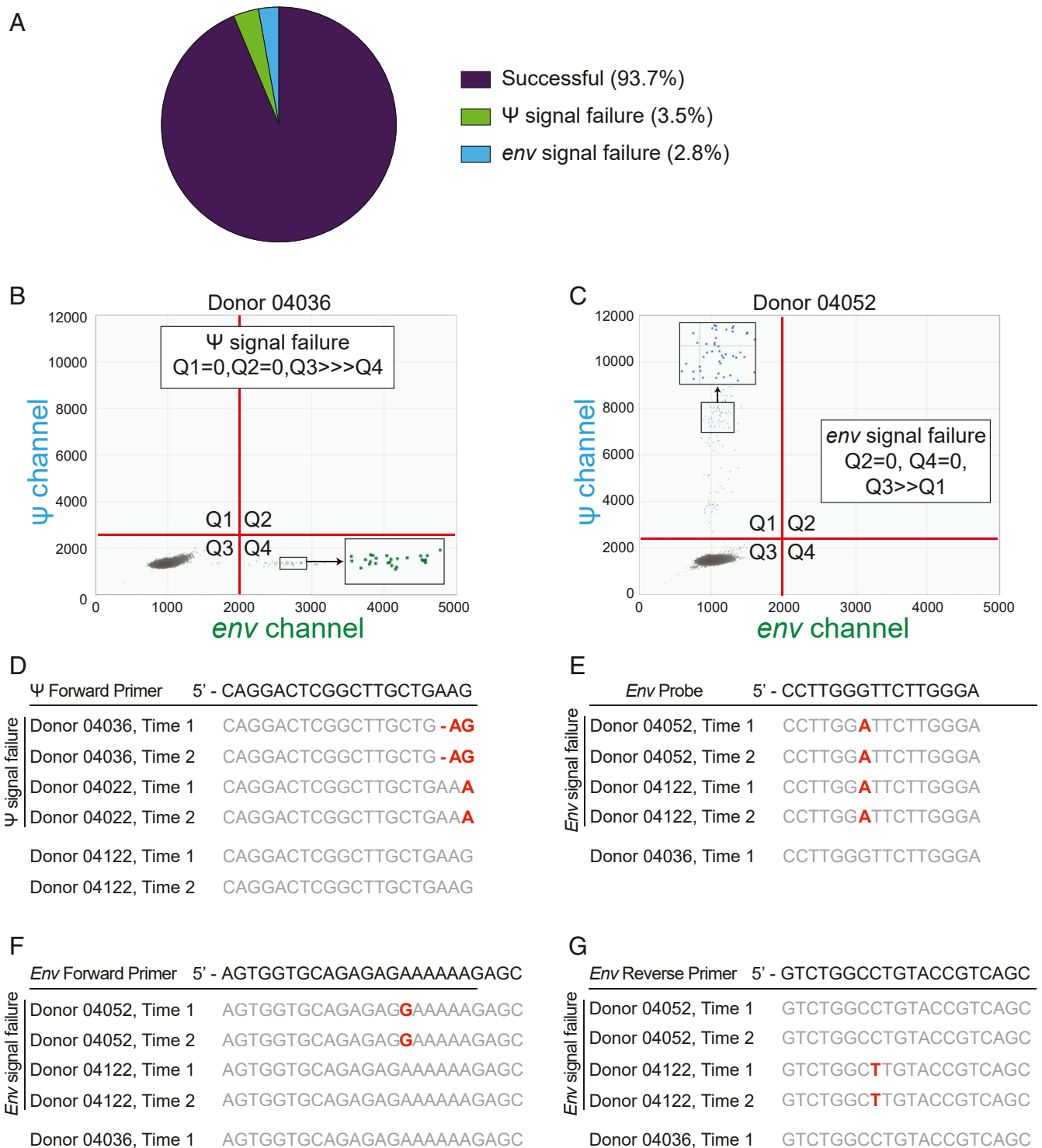


Fig. 3. IPDA amplicon signal failure due to polymorphisms in primer or probe binding sites is infrequent. (A) Frequency of amplicon signal failure for the Ψ and *env* amplicons. Of the 400 individuals analyzed in this study, we observed Ψ amplicon signal failure in samples from 3.5% and *env* amplicon signal failure in samples from 2.8%. (B) Representative 2D ddPCR plot from an HIV-1 proviral discrimination reaction illustrating Ψ amplicon signal failure. Ψ amplicon signal failure is easily identified by the absence of droplets in Q1 and Q2. (C) Representative 2D ddPCR plot from an HIV-1 proviral discrimination reaction illustrating *env* amplicon signal failure, which is readily identified by the absence of droplets in Q2 and in Q4. (D–G) Targeted sequencing (*Methods*) in individuals with signal failure revealing polymorphisms that account for a lack of signal. Polymorphisms at the 3' end of the Ψ forward primer account for the Ψ signal failure observed in donor 04036 (B) and another donor, 04022. Donor 04052 (C) and another donor, 04122, showed normal Ψ amplification but failed *env* amplification. In these cases, polymorphisms in the *env* primers and probe were responsible. Donor 04036 showed no polymorphisms in this region and a normal droplet distribution.

are required to induce some replication-competent proviruses to produce sufficient virus to establish a spreading infection *in vitro* (5, 6, 36), which may reflect proviral silencing by epigenetic mechanisms (37, 38) or transcriptional interference from surrounding host genes (39, 40). The degree to which these mechanisms influence reactivation of latent proviruses *in vivo*, and whether such silencing may be permanent for some proviruses, remain unclear. The IPDA represents a novel approach to reservoir evaluation in that it quantitates intact proviruses regardless of their transcriptional status in any *in vitro* assay.

For the reasons stated above, the IPDA represents a significant advance in reservoir measurement. It has already proven useful in documenting factors associated with more rapid reservoir decay (19), differential decay rates of intact vs. defective proviruses (9, 19), and an inflection in the decay curve in individuals with HIV-1 on long term ART (19). As with all PCR assays for HIV-1, sequence polymorphisms that affect performance of the IPDA will be observed. In the present study, 6.3% of individuals exhibited amplicon signal failure resulting in nonreportable IPDA results. Through targeted sequencing, we were able to demonstrate that these amplicon signal failures are a result of polymorphisms. Importantly, we show that these polymorphisms are stable over time in individuals on suppressive ART.

An advantage of the IPDA is that problems created by these sequence polymorphisms are readily apparent as an absence or dramatically reduced number of positive droplets for either the ψ or *env* amplicons. Importantly, the IPDA does not generate a false-negative result in these situations, because the presence of infected cells is indicated by the other amplicon. In contrast, for single amplicon assays, it is impossible to distinguish amplicon failure due to polymorphisms from low template concentrations.

While continued interrogation of large and diverse cohorts of infected individuals with the IPDA is likely to reveal additional unique sequence polymorphisms that interfere with amplification, this study provides an important performance benchmark for the assay. Alternative sets of primers and probes allow measurement of intact proviruses in cases of amplicon failure, as illustrated in Fig. 4. Alternative primers and probes that will allow successful amplification in individuals with the common polymorphisms are currently under development and should alleviate this problem in most cases.

In conclusion, a scalable assay for quantifying intact HIV-1 proviruses is essential for the evaluation of cure strategies. The IPDA provides a novel approach for reservoir quantitation that allows for a much more accurate assessment of reservoir size than standard PCR assays and does not depend on inefficient long-distance PCR. Analysis of samples from a large and diverse set of infected individuals shows that the frequency of proviruses lacking major common defects is on average 50 to 60 per 10^6 CD4⁺ T cells, 1 to 2 logs higher than the frequency detected with the QVOA and consistent with previous studies demonstrating that the QVOA underestimates the true size of the latent reservoir.

Methods

Study Participants and Samples. Samples were obtained from six independent cohorts of adults with HIV-1 on ART living in the United States (Table 1). The aggregated study population was diverse with respect to age, sex, race, HIV risk factors, and other clinical parameters. Details of each contributing cohort will be reported separately. Deidentified, previously collected samples were provided to AccelevirDx in the form of cryopreserved peripheral blood mononuclear cells (PBMCs). Each sample source confirmed that Institutional Review Board approval had been obtained for each study cohort, and that each individual had provided informed consent at the time of enrollment.

IPDA Measurements. In-depth descriptions of the IPDA rationale and procedure have been published previously (9, 19). In this study, the IPDA was performed by AccelevirDx under company standard operating procedures on DNA from CD4⁺ T cells isolated from cryopreserved PBMCs. As described previously (9), each IPDA consists of two multiplex ddPCR reactions performed in parallel: the HIV-1 Proviral Discrimination reaction, which distinguishes intact from defective proviruses (Fig. 1A); and the Copy Reference/Shearing reaction, which quantifies DNA shearing and input diploid cell equivalents. In this study, we observed a mean and median DNA shearing index (DSI) of 0.38, consistent with our previous report (9). The shearing correction is applied to both IPDA component reactions to account for the measured intra-amplicon shearing using the DSI, and final IPDA results are reported as proviral frequencies per million CD4⁺ T cells. ddPCR reactions were assembled via automated liquid handlers to maximize reproducibility. Samples were batch-processed and analyzed. For each batch, CD4⁺ T cells from uninfected donors were parallel-processed and analyzed as negative controls, while cells of the JLat full-length clone 6.3 served as positive controls. Clone 6.3 from E. Verdin was obtained through the NIH AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. Operators were blinded to donor identity and time point.

Targeted Proviral Sequencing. Targeted proviral sequencing was performed to obtain IPDA amplicon sequences. To obtain material for sequencing, bulk subgenomic PCR was performed on gDNA extracted from CD4⁺ T cells of HIV-1-infected individuals of interest to amplify regions of the proviral genome containing each IPDA amplicon. Sequencing was performed by independent approaches to verify results.

In some instances, a nested PCR approach was used to amplify these regions. For nested PCR reactions, amplification was performed with the Q5 High-Fidelity PCR Kit (New England BioLabs) with primer concentrations of 0.5 μ M. The outer PCR product was diluted 1:1,000 in 10 mM Tris before inner PCR amplification. PCR cleanup of inner PCR reactions was performed using the PureLink PCR Purification Kit (Invitrogen), and the presence of a single PCR amplicon of correct size was verified by agarose gel electrophoresis. Proviral sequencing of inner PCR products was performed by either traditional Sanger sequencing or short-read next-generation sequencing (Amplicon-EZ; GENEWIZ). Sequences were aligned to the HXB2 reference genome (Los Alamos National Laboratory HIV Sequence Database; accession no. K03455.1 GI:1906382) using Geneious Prime to identify polymorphisms.

In other instances, nested PCR amplification was performed with the Platinum Taq DNA Polymerase High-Fidelity PCR Kit (Invitrogen) with primer concentrations of 0.5 μ M. The outer PCR product was diluted 1:45 in 5 mM Tris before inner PCR amplification. PCR cleanup of inner PCR reactions was performed using the KAPA Pure Beads Kit (Roche), and the presence of a single PCR amplicon of correct size was verified by agarose gel electrophoresis. Proviral sequencing of inner PCR products was performed by Sanger sequencing (GENEWIZ). Sequences were aligned and analyzed for quality using Sequencher 5.4.

Data Availability. All experimental results described in the paper are presented in the table and figures. The IPDA method has been previously described in detail (9, 19). A description of study cohorts is given in Table 1. Detailed descriptions of each cohort have been published (19, 21, 24) or will be published separately and can be made available on request.

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