The Intact Proviral DNA Assay (IPDA) was developed to address the critical need for a scalable method for intact HIV-1 reservoir quantification. This droplet digital PCR-based assay simultaneously targets two HIV-1 regions to distinguish genomically intact proviruses against a large background of defective ones, and its application has yielded insights into HIV-1 persistence. Reports of assay failures however, attributed to HIV-1 polymorphism, have recently emerged. Here, we describe a diverse North American cohort of people with HIV-1 subtype B, where the IPDA yielded a failure rate of 28% due to viral polymorphism. We further demonstrate that within-host HIV-1 diversity can lead the IPDA to underestimate intact reservoir size, and provide examples of how this phenomenon could lead to erroneous interpretation of clinical trial data. While the IPDA represents a major methodological advance, HIV-1 diversity should be addressed before its widespread adoption as a principal readout in HIV-1 remission trials.
The Intact Proviral DNA Assay (IPDA) was developed to address the critical need for a precise and scalable method to quantify intact HIV-1 proviruses, which represent the main barrier to achieving HIV-1 remission or cure. This duplexed droplet digital PCR (ddPCR) assay simultaneously targets two HIV-1 regions, the Packaging Signal (Ψ) near the 5′ end of the viral genome and the Rev Responsive Element (RRE) within Envelope (env), to distinguish genomically intact proviruses against a large background of defective ones. The IPDA requires less time, resources and biological material than the current gold standard for replication-competent HIV-1 reservoir measurement, the Quantitative Viral Outgrowth Assay (QVOA), while also quantifying the total proviral burden. Application of the assay to increasing numbers of individuals living with HIV-1 has yielded insights into reservoir composition and dynamics, and the assay is being recommended for use in clinical trials evaluating HIV-1 remission strategies.

Recently however, instances of assay failure, attributable to natural HIV-1 polymorphism in primer and/or probe binding regions, have been described. While detection failures are expected for any molecular assay targeting a genetically variable pathogen, this rate has varied markedly across studies, ranging from 0% in the initial two reports (0/621 and 0/814) to 12% (6/50) in the most recent publication, where the assay was performed by the same group. A recent multi-cohort study comprising 400 individuals reports an overall 6.3% failure rate; however, because this study includes the above and other published cohorts with 0% failure rates, it can be inferred that the rates for the unpublished datasets are higher. Thus, while the IPDA is undeniably a major methodological advance, further study is needed to delineate the impact of HIV-1 sequence diversity. This is particularly relevant to clinical trials, which would either require an accurate anticipated rate of failure for power calculations, or strategies to address diversity directly, which might include pre-enrollment screening to determine IPDA-detectability of an individual’s virus and/or development of secondary primers and probes.

Here, we aimed to further develop the IPDA by assessing its performance within a diverse cohort of individuals with HIV-1 subtype B from across North America. Our study is unique in a number of ways. First, with the exception of one study that investigated a subset (20%) of assay failures by HIV-1 sequencing, no studies to date have combined IPDA, QVOA, and HIV sequencing to examine the frequency and potential implications of assay failures in detail. We describe an IPDA failure rate of 28% in our cohort, where we used QVOA to confirm that intact proviruses were indeed present in these cases, and where we present the underlying sequence polymorphisms for each case of failure. Second, we illustrate how within-host HIV diversity—specifically, cases where an individual harbors viral variants that are detectable and others that are undetectable by the assay—could lead the IPDA to underestimate intact HIV-1 reservoir size. We further provide examples of how this type of error could negatively impact clinical trial results interpretation. Third, our study provides an assessment of the IPDA by a group other than its original developers (or the associated company Accelevir Diagnostics). This could benefit the field, given that a major advantage of the IPDA is its potential for broad accessibility. Our failure rate of 28% occurred in the context of our efforts to precisely match the reported experimental conditions, with one exception which could have affected a minority of these cases. Whereas Peluso et al. state that gates to define positive droplets can be drawn tightly against double-negative droplets as defined in samples from individuals without HIV-1, we demonstrate that fluorescence signal from Ψ-positive templates (labeled with FAM) can bleed into the env channel, creating a false double-positive population, if gated in this manner. Thus, we required separation between negative and positive droplet populations in order to consider a result valid. This represents a previously unreported source of error in the IPDA. Taken together, we present an independent assessment of the scope of the challenge of addressing HIV-1 diversity in the IPDA, and—through sequence characterization—contribute to understanding the catalogue of polymorphisms that will need to be addressed with secondary primers and probes, towards improved iterations of this valuable assay.

**Results**

**IPDA measurements in a diverse North American cohort.** We applied the IPDA to 46 individuals with HIV-1 subtype B who had achieved viral suppression from cohorts across Canada, the USA and Mexico. Initial assay results yielded a median of 29 (interquartile range [IQR] 0-93) intact proviruses/million CD4+ T-cells (Supplementary Fig. 1); however, among these were 17 participants (37%) for whom the IPDA did not detect any intact (i.e., Ψ and env double-positive) proviruses. In four of these individuals, both Ψ- and env- single-positive proviruses were detected, suggesting a true-negative result (see example in Fig. 1a and Supplementary Fig. 2). In the remaining 13 individuals, however, the IPDA did not detect Ψ- and/or env- single-positive proviruses above background levels and instead yielded ddPCR plots that typify assay failure (Fig. 1a, Supplementary Fig. 2). Specifically, in eight of these participants only Ψ-positive proviruses were detected, in four only env-positive proviruses were detected, and in one participant no proviruses were detected. Furthermore, replication-competent HIV-1 was recovered in 11/11 (100%) cases where sufficient biological material was available to perform QVOA, consistent with the failure of IPDA to detect autologous intact proviruses.

**HIV sequence polymorphism can cause IPDA detection failure.** We next investigated the presumed cases of IPDA failure using HIV-1 sequencing. Near-full-length single-genome proviral sequencing performed on IPDA env-negative participant BC-004 revealed mismatches to the IPDA env probe, where in silico predicted reservoir distributions that took these polymorphisms into account (Fig. 1b, left) differed markedly from the original experimentally-obtained result (Fig. 1b, center). Substituting an autologous env probe rescued detection to in silico predicted levels (Fig. 1b, right), confirming that HIV-1 polymorphism can cause the IPDA to fail. Mismatches in the probe and/or at the 3′ end of a primer were confirmed in all 13 cases of presumed assay detection failure (Fig. 1c). Together, this yielded an overall failure rate of 13/46 (28%). Indeed, exclusion of these 13 datapoints markedly improved the correlation between IPDA and QVOA results among those for whom sufficient biological material was available to perform the latter assay, from r = 0.03, p = 0.83 to r = 0.35, p = 0.07 (Fig. 1d), bringing this correlation more in line with that reported by the original authors and what is expected biologically. By contrast, the correlation between QVOA and total HIV-1 gag DNA, measured using different HIV-1 primers and probe located in a conserved region, was significant without exclusion of these datapoints (Fig. 1e).

**Within-host HIV diversity can cause reservoir underestimation.** Although laborious to correct, complete detection failures due to HIV-1 polymorphism are nevertheless easy to flag due to their characteristic ddPCR plot presentation (Fig. 1a, Supplementary Fig. 2). In contrast, within-host HIV-1 diversity in IPDA primer or probe regions (see Fig. 1b for an example of
diversity within a probe region) could lead the IPDA to underestimate intact reservoir size if the within-host variants were differentially detectable by the assay. Such partial detection failures would not be easy to identify. Moreover, if IPDA-detectable and nondetectable reservoir subpopulations were differentially susceptible to HIV-1 cure interventions, this could lead to erroneous conclusions regarding intervention efficacy.

Although such a scenario remains hypothetical, we nevertheless illustrate our point using HIV-1-specific broadly neutralizing antibodies (bNabs) as an example. bNabs can facilitate elimination of HIV-1-infected cells\textsuperscript{12} in part by targeting them for antibody-dependent cellular cytotoxicity (ADCC)\textsuperscript{12,13}. As such, they are being evaluated in clinical trials, some of which use the IPDA as a readout (e.g., ACTG A5386\textsuperscript{14}). Participant 91C33 from a published trial\textsuperscript{15}
provides a hypothetical example. This individual did not respond to (off-Art) infusions of the bNabs 3BNC117 and 10-1074 because they harbored a plasma HIV-1 subpopulation that was resistant to both bNabs\(^{15}\) (Fig. 2a). Of note, this plasma HIV-1 subpopulation also harbored a mismatch to the IPDA env probe that we predicted would cause signal failure. We experimentally confirmed that, when using the published conditions, the IPDA could not detect templates harboring this polymorphism (Fig. 2b), though signal could be detected if the annealing/extension temperature was lowered to harboring this polymorphism (Fig. 2b), though signal could be detected if the annealing/extension temperature was lowered to 53°C to account for the mismatch (Supplementary Fig. 3). In contrast, templates representing the bNab-sensitive strains could be detected readily using the published conditions (Fig. 2b). Theoretically, if a person harboring such diversity in their reservoir were to be successfully treated with one or both bNabs in a trial that used the IPDA as the sole readout, the intervention’s effect on reducing the reservoir would be overestimated because the assay was only capable of detecting the susceptible reservoir fraction (Fig. 2e, left).

ART-suppressed participant OM5346 provides another example. Pre-Art HIV-1 drug resistance genotyping identified subtype B infection; however, single-genome sequencing of pre-Art plasma and proviruses sampled during long-term Art revealed co-infection with a non-B strain (Supplementary Fig. 4). While the IPDA is only designed for subtype B, the co-infecting strain harbored no critical mismatches within IPDA target regions except for a G-to-A mismatch at position 13 (G13A) of the env probe. Importantly, G13A is amongst the most frequent probe polymorphisms in subtype B (and was observed in 2/10 study participants for whom env was sequenced), and the original report indicated that the IPDA was capable of detecting env sequences harboring it as the sole variant, at least when present on a plasmid template\(^{4}\). As such, the assay should be able to detect this strain despite its non-B status. Using QVOA, we successfully isolated replication-competent subtype B (virus 3) and co-infecting (virus 4) strains from OM5346’s reservoir, confirming both of these as true intended targets of the IPDA (Fig. 2c). We further observed that these viruses were differentially susceptible to 3BNC117- and 10-1074-mediated ADCC: while virus 3-infected cells could be eliminated by 10-1074, but not 3BNC117-, mediated ADCC (25% and 0% relative reduction, respectively), the opposite was true for virus 4 (0% and 32% relative reduction, respectively) (Fig. 2c, Supplementary Fig. 5). We further confirmed that, while the IPDA could readily detect provirus 3 at concentrations approximating biological samples, the assay could not distinguish provirus 4 at these concentrations (Fig. 2d, Supplementary Fig. 6a). Similarly, at biological concentrations, the IPDA could not detect a synthetic DNA template encoding the virus 4 sequence, though noticeable signal elevation occurred at ~100-fold higher template concentrations (Supplementary Fig. 6b). Importantly, a secondary env primer/probe set (see below and methods) readily detected both viruses at all concentrations, regardless of template type (Supplementary Fig. 6). In the hypothetical case where this individual were to be successfully treated with a bNab in a trial that used IPDA as a sole readout, different erroneous interpretations could occur depending on the intervention used. Specifically, if this individual were to be successfully treated with 10-1074, the IPDA would overestimate the intervention’s effect on the reservoir because the assay was only capable of detecting the susceptible reservoir fraction. In contrast, if the individual were to be successfully treated with 3BNC117, the IPDA would erroneously conclude that the intervention had no effect because the assay was only capable of detecting the resistant reservoir fraction (Fig. 2e, right).

Optical bleed-through as a source of error in the IPDA. Although HIV-1 polymorphism can clearly cause the IPDA to fail in some instances, certain mismatches can be tolerated\(^{45}\). This is due to the assay’s use of endpoint (rather than real-time) PCR detection, such that polymorphisms that reduce signal can yield lower-amplitude droplets that can nevertheless still be scored as positive, provided that they are sufficiently separated from the negative population. In fact, participant OM5346’s two viral populations illustrate this phenomenon: virus 3, which harbors a single nucleotide deletion as well as a mismatch in the env probe, produces modest yet clearly discernible signal in the env channel, whereas virus 4, which also harbors minor primer/probe mismatches, produces higher-amplitude env signal (Fig. 3). Detailed investigation of such instances of HIV-1 sequence-driven amplitude modulations led us to identify another source of potential error, in the form of optical bleed-through, a challenge in multiplexed digital PCR assays\(^{16}\). This occurs when fluorescence from the channel, which features the FAM fluorophore, bleeds into the env channel, which features the VIC fluorophore, to varying extents. This in turn has implications for the appropriate placement of thresholds defining negative and positive populations. Critically, because the extent of bleed-through is determined by the amplitude of the env signal, which is in turn determined by the extent of match to the assay probe, the extent of fluorescence spillover is HIV-1 sequence-specific. We demonstrate this by applying the IPDA to synthetic templates encoding the env regions of OM5346 viruses 3 and 4, without a corresponding env template present (Fig. 3). Virus 3’s env signal, which is modest, does not bleed into the env channel. By contrast, virus 4’s high amplitude env signal bleeds noticeably into the env channel. Thus, whereas a tight threshold based on the negative
population can be drawn for virus 3, doing so for virus 4 creates env signal (and in fact intact provirus signal) even when no env template is present. This \( \Psi \) channel bleed-through for virus 4 thus likely contributed to the small amount of env signal in Fig. 2d (events adjoining the negative population). While ddPCR instruments are factory-tuned to mitigate spectral overlap, this basic property of fluorophores underscores the instrument manufacturer’s instruction that appropriate thresholds can only be drawn if negative and positive populations clearly separate from one another\(^{17}\). Similarly, it cautions against the setting of gates solely based on data from uninfected donors, as suggested by Peluso et al.\(^4\) as a strategy for suboptimal amplifications.

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**Table 1:**

| Virus | IPDA Env Probe (nucleotide seq.) |
|-------|----------------------------------|
| 91C33 | gpl20 codon 280 281 332 334 |
| MH632930 | N A S S |
| MH632931 | - - - - |
| MH632932 | - - - - |
| MH632933 | - - - - |
| MH632934 | - - - - |
| MH632935 | - - - - |
| MH632936 | - - - - |
| MH632937 | - - - - |
| MH632938 | - - - - |
| MH632939 | - - - - |
| MH632940 | - - - - |
| MH632941 | - - - - |
| MH632942 | - - - - |
| MH632943 | - - - - |
| MH632944 | - - - - |
| MH632945 | - - - - |
| MH632946 | - - - - |
| MH632947 | - - - - |
| MH632948 | - - - - |
| MH632951 | - - - - |
| MH632954 | - - - - |
| MH632955 | - - - - |
| MH632949 | S H T S c |
| MH632950 | S H T S c |
| MH632952 | S H T S c |
| MH632953 | S H T S c |

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**Figure 2:**

(a) Table of IPDA Env Probe (nucleotide seq.) for virus 3 and 4.

(b) Graph showing the effect of input DNA concentration on the measured concentration of IPDA.

(c) Graph showing the percentage of infected cells remaining relative to no Ab control for virus 3 and 4.

(d) Graph showing the effect of input DNA concentration on the measured concentration of IPDA.

(e) Intra-individual HIV Diversity: Impact on Intervention Evaluation by IPDA.
Addressing interhost HIV diversity in the IPDA. The IPDA offers major scalability and accessibility advantages over existing molecular or culture-based approaches, and a recent study that employed a quadruplexed qPCR assay confirmed that, of all probes evaluated, those used in the IPDA offered the greatest selectivity for intact proviruses. Unfortunately, the requirement to discriminate proviral defects in the Ψ region restricts the options for placement and sequence of these oligonucleotides, and the limited data currently available for Ψ region polymorphisms in IPDA failure cases (Fig. 1c and ref. 5) reveal few common polymorphisms that could be used to design secondary primer/probe sets. By contrast, placement of the 3′ target is less constrained. This allowed us to develop a secondary primer/probe set in the intact-discriminating RRE region, approximately 50 bases downstream of the original location, as a first step towards addressing HIV-1 diversity with a strategy other than autologous oligonucleotide design or data exclusion. This primer/probe set rescued detection of env-positive proviruses in 9/9 participants with IPDA env detection failure (Fig. 4a). When applied to the 36 participants for whom the IPDA detected env-positive proviruses (excluding OM5346 who harbored within-host diversity), it failed to detect env-positive proviruses in 3 (8%) individuals, indicating that it is not a universal solution. For the remaining 33 (92%), however, it yielded measurements that correlated strongly with those from the IPDA (Fig. 4b). The secondary primer/probe set could therefore be used to confirm instances of detection failure without confirmatory viral sequencing as well as to estimate intact provirus levels, though users must note that, unlike the IPDA env reaction, it cannot discriminate hypermutated sequences.

Discussion
The IPDA is undoubtedly a major advance in HIV-1 persistence research, but recent reports of variable and increasing failure rates due to HIV-1 polymorphism are concerning. While the original and early follow-up studies acknowledged HIV-1 diversity as a potential limitation that would require custom primer/probe sets, neither study reported any failures due to viral polymorphism (out of 62 and 81 participants, respectively). More recent studies of 61 and 50 participants reported failure rates of 9.8% and 12%, respectively. The largest study undertaken to date using the IPDA, a multi-cohort study published by the assay’s developers, reports an overall failure rate of 6.3% of 400 participants, although it is important to note that this study includes a number of published cohorts including four with 0% failure rates. As such, the inferred failure rate for some of the unpublished datasets could be as high as 18%, a value that approaches the 28% rate reported in the present study.

Taken together, our findings indicate that as the IPDA is applied to diverse cohorts, detection failures will occur, and not infrequently. The data also suggest that failure rates may vary markedly by cohort, presumably due to local HIV-1 diversity in regions where HIV-1 subtype B circulates. Indeed, failure rates differed markedly between regions in our own study: whereas the failure rates in the Vancouver and Toronto cohorts were 2/15 (13%) and 3/19 (16%) respectively, those in the Washington, D.C. and Bronx, N.Y. cohorts were 4/7 (57%) and 3/4 (75%) respectively. Moreover, these failures were not attributable to shared HIV-1 polymorphisms nor to epidemiologic linkage (Fig. 1c), and all infections were HIV-1 subtype B.

The first step towards mitigating this issue is to raise awareness: samples that yield no Ψ or env single-positive proviruses should be flagged as unreportable until HIV-1 polymorphism has been addressed (e.g., using autologous primers/probes). Nevertheless, elevated failure rates limit the assay’s scalability and utility: autologous probe design is time-consuming and costly, and exclusion of assay failures could bias study results if failure rates are substantial. Our findings therefore underscore the importance of better understanding the frequency and nature of HIV-1 polymorphisms within IPDA target regions and their impact on intact proviral quantification. Analysis of HIV-1 subtype B sequences from unique individuals in the Los Alamos HIV database revealed that 23% of 9360 env sequences harbored at least one env probe mismatch (which is similar to our env detection failure rate of 9/46 or 20%), while 50% of 1489 sequences harbored at least one env probe mismatch (which is substantially greater than our env detection failure rate of 5/46 or 11%). This suggests that the env reaction may be more sensitive to polymorphism than the Ψ reaction, but larger studies linking proviral sequence to IPDA readouts are needed to confirm this.

Similarly, due to a paucity of studies that pair detailed within-host sequence characterization with IPDA measurements, it is not currently possible to estimate the extent and impact of within-host HIV-1 diversity on intact reservoir quantification by the IPDA. Nevertheless, when taken together with Gaebler et al.’s observation that four of nine (44%) studied reservoirs were heterogeneous in an IPDA probe region, our combined observations suggest that the impact of within-host HIV-1 diversity on IPDA accuracy may be non-negligible.

In conclusion, we provide an independent assessment of HIV-1 sequence diversity as a source of error in the IPDA by integrating IPDA, QVOA, and viral sequence data to characterize detection
failures. We report a substantially higher rate of complete detection failures than previous studies, and additionally identify partial detection failures, attributable to within-host viral variation, as a source of error in the IPDA. The latter issue is particularly challenging given that it is essentially impossible to detect without HIV-1 sequencing, but given the reservoir’s highly dynamic nature and our identification of cases where within-host diversity is linked to HIV-1 cure intervention susceptibility, it should not be ignored. Given the clear value of the IPDA, iterative efforts to refine this assay are a priority. In the meantime, we recommend that proviral sequencing, at minimum within the IPDA target regions, should be performed for participants of HIV-1 remission clinical trials in which the IPDA is employed, and secondary and/or autologous primers/probes used to mitigate HIV-1 diversity.
Fig. 3 HIV sequence-specific optical bleed-through prohibits tight thresholding on negative populations. a 2D and env 1D plots of OM5346 virus 3’ env region sequence when tested as a synthetic DNA gene fragment (Virus 3’ env gagblock) without corresponding env template. Minimal env- to env- channel spillover occurs and the positive droplet threshold can be drawn tightly to the double-negative population without consequence (note the presence of one false double-positive droplet). b 2D and env 1D plots of OM5346 virus 4’ env region sequence, tested as a synthetic DNA gene fragment (Virus 4’ env gagblock), without corresponding env template. Drawing a tight threshold causes optical bleed-through of env (FAM) fluorescence into the env (VIC) channel to yield a false-positive env (and by extension, false-positive intact) signal. c 2D and env 1D plots of OM5346 Virus 4’ env region sequence, tested as a synthetic DNA gene fragment (Virus 4’ env gagblock) without corresponding env template, with a threshold drawn at an appropriate distance from the double-negative population. This threshold accommodates the fluorescence shift and thus avoids the creation of false-positive intact or env-positive droplet population (note the presence of a single false double-positive droplet). Representative plots from one of three technical replicates from one experiment are shown.

Fig. 4 Performance of the secondary env primer/probe set. a A secondary env primer/probe set rescued detection in all 9 cases of IPDA env detection failure (p = 0.004, two-sided Wilcoxon signed-rank test). b A secondary env primer/probe set performs comparably to the IPDA in samples detectable by the latter assay. Black datapoints denote 33 participants whose reservoir was detectable by both IPDA and secondary env primer/probe sets; red datapoints denote three participants whose reservoir was detectable by IPDA but not using the secondary env primer/probe set. Excluding the latter three points yielded a Spearman’s p = 0.82, p < 0.0001 (shown on figure). Including these three red datapoints yielded a Spearman’s p = 0.68, p < 0.0001. Datapoints show point estimate from four merged technical replicates for each participant sample. Source data are provided in the Source Data File.

Methods
Participants and ethics statement. We studied 46 individuals with HIV-1 subtype B who were recruited to cohorts in Toronto (N = 19), Vancouver (N = 15), New York City (N = 4), Washington, D.C. (N = 7), and Mexico City (N = 1). For Vancouver participants, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation and cryopreserved (−150 °C, 90% Fetal Bovine Serum + 10% DMSO). Participants recruited at other sites provided a leukapheresis sample from which PBMC were isolated and cryopreserved as above. All participants were on long-term, virally suppressive combination antiretroviral therapy (cART) at time of sampling, with the exception of one elite controller. Participants’ duration of untreated HIV-1 infection ranged from 1 month to >10 years, though the exact duration was unknown for most participants. Ethical approval to conduct this study was obtained from the Institutional Review Boards of Simon Fraser University, Providence Health Care/University of British Columbia, Well Cornell Medicine, and the George Washington University. All participants provided written informed consent.

Quantitative Viral Outgrowth Assay (QVOA). For participants for whom sufficient biological material was available, the Quantitative Viral Outgrowth Assay (QVOA) was performed. CD4 + T-cells were isolated from PBMCs by negative selection and plated in serial dilution at either 4 or 6 concentrations (12 replicate wells/concentration, 24-well plates). CD4 + T-cells were stimulated with phytohemagglutinin (PHA, 2 μg/mL) and irradiated allogeneic HIV-1-negative PBMCs were added to further induce viral reactivation. MOLT-4/CCR5 cells were added at 24-h post-stimulation as targets for viral infection. Culture media (RPMI 1640 + 10% FBS + 1% Pen/Strep) was changed every 3 days and p24 enzyme-linked immunosorbent assay (ELISA) was run on day 14 to identify virus-positive wells. Infectious Units per Million CD4 + T-cells (IU/M) was determined using the Extreme Limiting Dilation Analysis (ELDA) software (version 1.0, http://bioinf.wehi.edu.au/software/elda). Culture supernatants from virus-positive wells were frozen (−80 °C) for future use.

Intact Proviral DNA Assay (IPDA). Genomic DNA was isolated from a median 7.5 ng (IQR 7–7.5 ng) (RPP30) or a median 750 ng (IQR 700–750 ng) (HIV-1) of genomic DNA was combined with ddPCR Supermix for Probes (no dUTPs, BioRad), primers (final concentration 900 nM, Integrated DNA Technologies), probe(s) (final concentration 250 nM, ThermoFisher Scientific) and nuclease free water. Primer and probe sequences (5’→3’) were: RPP30 Forward Primer- GATTTGGACCTGCGAGAGGTTCTATTGTAG- MGBNFQ, RPP30 Reverse Primer- GCCGGCTGTCTCCACAAGT; RPP30-Shear Forward Primer- CACATTTGCTGCTCTTCGGG, RPP30-Shear Reverse Probe- VIC-AGTGGTGCAGAGAGAAAAAGAGC, HIV-1 S Primer Probe- TTTGGGCTAATCTCACCAGT- MGBNFQ, HIV-1 S Reverse Primer- GCACCCATCTCTCTCTCTCTGAG; HIV-1 env Forward Primer- AGGATGGTGACAGAGAGAAAAAGAGC, HIV-1 env Probe- VIC-CTTGGGTTCCTGCTTCGGG, HIV-1 env Reverse Primer- GCACCCATCTCTCTCTCTCTGAG; MGBNFQ, anti-Hypermutant env Probe- CTCAGTGCTCTAGAGGCACC, MGBNFQ, HIV-1 env Reverse Primer- GTGCAGCTCTCTCTCTCTGAG; MGBNFQ, HIV-1 env Primer- VIC-CTTGGGTTCCTGCTTCGGG, HIV-1 env Probe- VIC-CTTGGGTTCCTGCTTCGGG.
and probe (5′→3′): HIV-1 gag Forward—CTCTGACACGGACTGCG; HIV-1 gag Reverse—CTCTGACCTCTGCCACCC; HIV-1 gag Probe—FAM—CTCTGCTCT/2ZEN/TCCTGCTCT; 2ZEN (IDT). Reactions were cycled as follows: 40 cycles of (94 °C for 30 sec, 53 °C for 1 min) and 98 °C for 10 min. A minimum of four (maximum eight) technical replicates were performed for each sample, where data from replicate wells was merged prior to analysis.

**HIV-1 proviral amplification and sequencing.** Single-template, near-full-length proviral DNA was extracted from D4 + T-cells by nested PCR using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) such that ~25% of the resulting PCR reactions yielded an amplicon. First round primers were: Forward—AAATCTTCAGTGAGGGCGCAAGAC, Reverse—TGAAGGG ATCTCTAGTACCGAGTC. Second round primers were: Forward—GC GCCGAACAGGACCTGGTAAAC, Reverse—GACTGCAAGGCA AGCTTTATGAGCCCTA. Reactions were cycled as follows: 92 °C for 2 min; 10 cycles of (92 °C for 10 sec, 60 °C for 30 sec, and 68 °C for 10 min); 20 cycles of (92 °C for 10 sec, 55 °C for 30 sec, and 68 °C for 10 min); 68 °C for 10 min.2, 22, 23, Amplicons were sequenced using Illumina MiSeq technology and de novo assembled using the custom software MiCall (https://github.com/cfe-lab/MiCall) which features an in-house modification of the Iterative Virus Assembler (IVA)24, or through collaboration with the Massachusetts General Hospital CGIB Core.

**In silico predicted IPDA results.** Linked V and env probe region sequences were excised from BC-004 proviral sequences for in silico prediction of IPDA results under the assumption that intact probe regions would be detected by the assay. A V or env probe region sequence was considered defective if it contained mutations associated with defective proviruses (e.g., those consistent with hypermutation1 or common splice donor site mutations15) or if it was completely absent. All other probe region sequences, regardless of whether they matched the published IPDA probe sequence, were considered intact.

**Targeted HIV-1 sequencing.** For participant OM5346, HIV-1 RNA was extracted from QVOA outlet supernatant and pre-ART plasma, after which qPCR was used to detect HIV RNA. Amplicons were sequenced using either Sanger (3730xl, Applied BioSystems) or Next Generation Sequencing (MiSeq, Illumina) which features an in-house modification of the Iterative Virus Assembler (IVA)24. Reactions were cycled at: 95 °C for 10 min; 40 cycles of (94 °C for 30 sec, 53 °C for 1 min) and 98 °C for 10 min. A minimum of four (maximum eight) technical replicates were performed for each sample, where data from replicate wells was merged prior to analysis.

**Antibody-dependent cellular cytotoxicity (ADCC) assays.** Total CD4 + T-cells were isolated from peripheral blood mononuclear cells (PBMCs) by negative selection using biotinylated antibodies to CD14, CD19, and CD56 (Miltenyi Biotec, Auburn, CA), followed by magnetic bead separation using EasySep (StemCell Technologies). CD4 + T-cells were stimulated with plate-bound anti-CD3 antibodies (Biolegend, catalogue number: 100950, 1 μg/ml) for 2 h. Natural Killer (NK) cells were negatively selected from PBMCs using negative selection columns (EasySep, StemCell Technologies) from PBMCs of allogeneic, HIV-1-negative donors and activated with anti-CD3 (Biolegend, catalogue number: 317326, clone: OKT3, 1:1000 dilution) and anti-CD28 antibodies (Biolegend, catalogue number: 302934, clone: CD282, 1:1000 dilution). Cells were then infected with the participant virus of interest collected from QVOA supernatant, and monitored by flow cytometry by intracellular staining for HIV-1 Gag. Negative controls were conducted using Coomasie Blue (Biolegend, catalogue number: 6046665, clone: KC57-RD1, 1:100 dilution) until cells were >5% Gag + infected cells remaining post-ADCC relative to no antibody control were then quantified using the following formula: (1 − (%Gag − cells in test − %Gag − cells in no antibody control))/%Gag − cells in no antibody control. %Gag − cells in no antibody control, %Gag − cells in test, %Gag − cells in no antibody control, and %Gag − cells in test were measured by flow cytometry using the secondary primer and probe locations. These were: Secondary primer CTCTGCTATTGACTGAC and probe sequence derived from clinical drug resistance testing (sampled in 2012), proviruses from purified CD4 + T-cells isolated during suppressive ART (sampled in 2017 and 2019) were sequenced using Sanger (3730xl, Applied BioSystems) or Next Generation Sequencing using MiSeq technology and de novo assembled using the custom software MiCall (https://github.com/cfe-lab/MiCall) which features an in-house modification of the Iterative Virus Assembler (IVA)24, or through collaboration with the Massachusetts General Hospital CGIB Core.

**Design of secondary env ddPCR primer/ probe set.** HIV-1 Subtype B envelope sequences, limited to one per individual, were retrieved from the HIV LAN database (N = 4670; https://www.hiv.lanl.gov/components/sequence/HIV/search/search.html) and aligned. Sequence conservation was assessed across 16–25 base-pair intervals and three intervals that maximizedsequence conservation while meeting ddPCR assay specifications (i.e., amplicon length, Tm) were chosen as secondary primer and probe loci. These were: Secondary env Forward Primer CATATGGGAGCAAGCTCTACAAGTG; Reverse Primer TCATTGATCACTAGCGATCTT (representing nucleotides 7809–7825 in the HIV-1 genome reference HXB2, predicted sequence conservation 79%). Secondary env Probe VIC-CTGGGCCTGACCTGCTAGC- MGRMNOQ (HXB2 nucleotides 7833–7849, 83% conservation). Secondary env Reverse Primer CCCAGAGATGAGTACCTG (HXB2 nucleotides 7939–7921, 88% conservation). Reaction composition and cycling conditions were identical to those for the IPDA as described above. Accurate quantification using the Secondary env primer/probe set was verified using DNA extracted from the J-Lat 9.2 cell line (obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, contributed by Dr. Eric Verdin)28 (Supplementary Fig. 7), where three independent experiments, each with four technical replicates, were performed and replicate wells were merged prior to data analysis.

**Statistical analyses.** All statistical analyses were performed using GraphPad Prism (version 8). All statistical tests were two-sided.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Data are available in the manuscript, Figures and Supplementary Figures. HIV sequences used for phylogenetic inference are available in GenBank under accession numbers MT592083–MT592223; HIV sequences for participant 91G3 were previously published under GenBank accession numbers MH613920–MH6329553. HIV-1 subtype B sequences used to identify the location for the secondary env primer/probe set are available through the Los Alamos National Laboratory HIV sequence database (https://www.hiv.lanl.gov/components/sequence/HIV/search/search.html). Additional information is available from the corresponding authors upon request. Source data are provided with this paper.

**Code availability**

Code for the custom software MiCall is available at https://github.com/cfe-lab/MiCall. Received: 1 September 2020; Accepted: 2 December 2020; Published online: 08 January 2021

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Author contributions

N.N.K., Y.R., Z.L.B., and R.B.J. designed the study. N.N.K., Y.R., W.D.C.A., W.D., P.K., S.H.H., T.M.M., A.S., and A.W. performed experiments. N.N.K., Y.R., W.D.C.A., W.D., P.K., S.H.H., T.M.M., A.S., A.W., D.K., C.J.B., G.Q.L., and R.M.L. analyzed data. M.H., S.H.H., T.M.M., A.S., and A.W. performed experiments. N.N.K., Y.R., Z.L.B., and R.B.J. wrote the manuscript. All authors contributed to the critical revision of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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